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The Role of the Cpx Two Component Regulatory System in Enteropathogenic *Escherichia coli* Pathogenesis

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of Master of Science

in

Microbiology and Biotechnology

Department of Biological Sciences

Edmonton, Alberta

Spring 2005

0-494-08172-4

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Abstract

The Cpx two component regulatory system and its regulated genes have been increasingly implicated in the assembly of virulence determinants in a number of pathogens. The subject of this thesis was to investigate the role of the Cpx envelope stress response in enteropathogenic *Escherichia coli* pathogenesis, a model organism with a number of cell envelope associated virulence determinants. All of the genes required for the formation of a characteristic histopathology known as attaching/effacing lesions reside on a chromosomal pathogenicity island known as the locus of enterocyte effacement (LEE). The LEE contains five polycistronic operons designated *LEE1* through *LEE5*. *LEE1*, *LEE2*, and *LEE3* encodes a type III secretory apparatus, *LEE4* encodes the translocated proteins, and *LEE5* encode an outer membrane adhesin, intimin, and its translocated receptor, Tir. Enzyme-linked immunosorbant assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the secreted virulence factors EspB and Tir revealed that when the Cpx pathway was activated, using either CpxR overexpression or the more physiologically relevant NlpE overexpression, there was an increase in both EspB and Tir. This elevated secretion resulted in increased hemolysis of sheep red blood cells, an indicator of a functional type III secretory system. The increase in secretion is partially transcriptional as *LEE2, LEE3* and *tir* undergo elevated transcription in response to Cpx activation, as determined by either RT-PCR or *lux* reporter analysis. The increase in secretion may reflect Cpx-regulated protein folding and degrading factors aiding in the folding of the type III-secretory system.

Acknowledgments

My time in the Raivio lab has been one of the most enjoyable periods of my life and an excellent academic experience. I am indebted to my supervisor, Tracy Raivio, for giving a "hungry" young undergraduate a chance in her lab and am grateful for her constant support, encouragement, and guidance. I really feel I grew as a scientist under her tutelage and learned so much from the international meetings that she allowed me to attend. I am also grateful to the members of my committee, Glen Armstrong and Brenda Leskiw, for their suggestions and help over the course of this thesis. I would also like to thank Rebekah DeVinney for reagents and instruction in pedestal formation, as well as for an interesting week in Calgary.

I'd like to express my gratitude to the entire Raivio lab past and present, particularly Chris Oates, Nancy Price, Daelynn Buelow, Dawn MacRitchie, and last but certainly not least, Anna Nevesinjac, for help and suggestions, but also for making the lab such an enjoyable places to be. I'll always fondly remember late night pizza, climbing, and swimming, as well as the various lab outings. I'd also like to acknowledge Jon Dennis and the members of the Dennis lab for being such a great neighbour lab, contributing to the great 3rd floor Micro atmosphere. It was certainly much easier to work long days and nights in such great environment. I would briefly like to thank my friends throughout the department, Ankush, Mike C., Mike H., Brian, Ryan, Rob and Sean, for support and banter.

Finally, I would like to thank my wife Elizabeth, my parents, my sister, and the rest of my family for their love and support. Their belief in me kept me going through some of the more difficult points of the thesis.

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List of abbreviations

A	absorbance
AE	attaching and effacing
Amp	Ampicillin
AP	alkaline phosphatase
Ara	Arabinose
BFP	bundle forming pili
bp	Basepairs
Cam	Chloramphenicol
CAP	catabolite activator protein
cat	chloramphenicol resistance gene+C49
cDNA	complementary deoxyribonucleic acid
Cpx*	gain of function Cpx mutant
CpxR-P	phosphorylated form of CpxR
C-terminal	carboxy terminal
DMEM	Dulbecco's modified Eagle media
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
Dnase	Deoxyribonuclease
EAF	EPEC adherence factor
ECA	enterobacterial common antigen
ECF	extracytoplasmic factor
EDTA	ethylenediaminetetraacetic acid
EHEC	enterohemorrhagic *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
GFP	green fluorescent protein
Glu	Glucose
IPTG	isopropyl-B-D-galactopyranoside
Kan	Kanamycin
kb	kilobase pair
kDa	Kilodalton
L	Litre
LB	Luria broth
LEE	locus of enterocyte effacement
LRP	leucine responsive protein
lux	*LuxCDABE*
Lux	Luciferase
M	Molar
mg	Milligram

mL	Millilitre
mM	Millimolar
mRNA	messenger ribonucleic acid
N-terminal	amino terminal
OD600	optical density
ORF	open reading frame
PAI	pathogenicity island
PBS	phosphobuffered saline
PCR	polymerase chain reaction
ppiase	peptidyl-prolyl isomerase
proteinase K	Protease
RBC	red blood cell
RNA	ribonucleic acid
RT	reverse transcriptase
SDS-PAGE	sodium dodecyl sulfide polyacrylamide gel electrophoresis
Spc	Spectinomycin
TAE	Tris-acetate EDTA
TCA	Trichloroacetic acid
Tet	Tetracycline
Tet^R	tetracycline resistance
Tris	(hydroxymethyl) aminomethane
TTS	Type III secretion
TTSS	Type III secretion system
UPEC	uropathogenic *Escherichia coli*
w/v	weight per volume
α	alpha, anti
B	Beta
Δ	delta, deletion
σ	Sigma
°C	degrees Celsius
μg	Microgram
μL	Microlitre
μM	Micromolar

Chapter 1

Introduction

1.1 Envelope Stress Responses

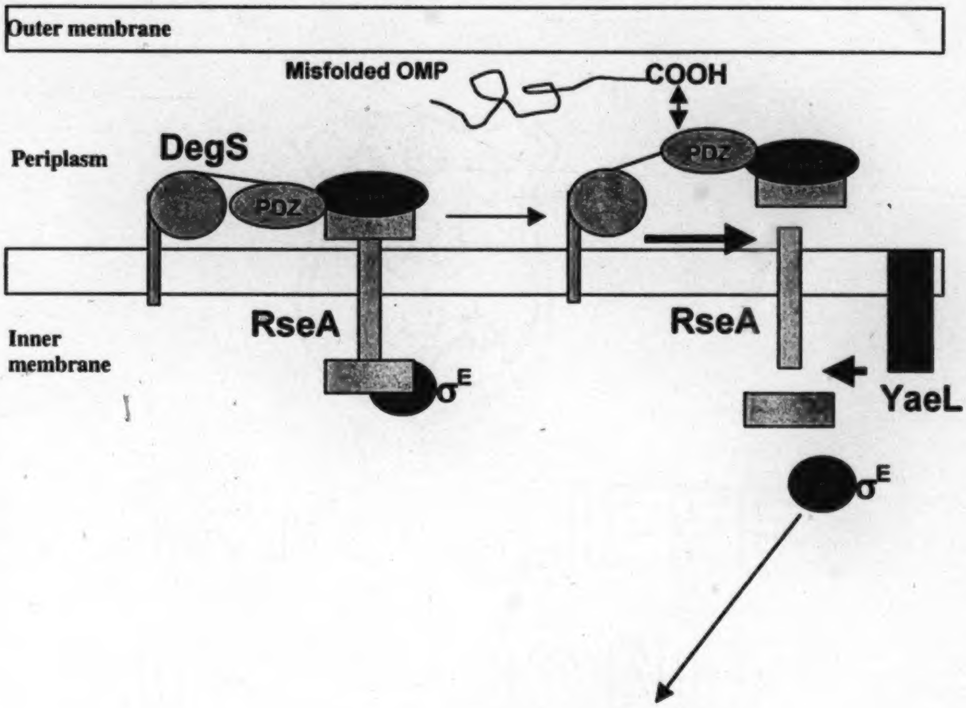
Bacteria are subject to a constantly changing environment and consequently must develop methods of sensing and adapting to stress. Bacteria can respond to stress in two ways; they can move away from the source of stress via motility and chemotaxis, or they can express stress combative genes. Hence the study of how bacteria respond to stresses such as ethanol, heat, and pH can give valuable insight into transcriptional regulation of stress-induced genes. Gram negative bacteria possess a cell envelope comprised of two lipid bilayers separated by a viscous solution of proteins and peptidoglycan in which proteins have been demonstrated to diffuse at a 100-fold lower rate than in the cytoplasm (Kadner, 1996; Oliver, 1996; Nikaido, 1996; Brass *et al.*, 1986). This envelope, in addition to serving as a protective barrier, is an oxidative compartment involved in solute transport, protein translocation, and lipid biosynthesis (Kadner, 1996; Oliver, 1996; Nikaido, 1996). Changes in the environment can cause perturbations to the membrane as well as protein denaturation, and cells respond to this envelope stress by upregulating the expression of various protein folding and degrading factors (for a review see Raivio and Silhavy, 1999). In *Escherichia coli* there are three distinct envelope stress responses: the σ^E pathway, the Cpx two-component regulatory system, and the Bae two-component regulatory system. The σ^E and the Cpx pathways both activate transcription of *degP*, a gene encoding a periplasmic serine endoprotease that digests misfolded envelope proteins (Erickson *et al.*, 1989; Danese *et al.*, 1995). The Cpx and Bae two-component systems are both positive regulators of *spy*;,which encodes a small periplasmic protein of unknown function (Raffa and Raivio, 2002).

1.1.2 The σ^E pathway

The σ^E system was the first envelope stress sensing system characterized in *E. coli*. The σ^E pathway belongs to the extracytoplasmic factor (ECF) family of sigma factors as it regulates an extracytoplasmic function (Erickson *et al.*, 1989). The ECF sigma factors generally use a membrane bound anti-sigma factor to transduce extracytoplasmic inducing cues to the cytoplasm (Raivio and Silhavy, 2001). σ^E was originally identified by its ability to activate transcription of the P3 promoter of *rpoH*, which encodes σ^H. This is a heat shock regulated promoter that is transcribed in a σ^{70}-independent manner. However σ^E was shown not to activate transcription of known σ^H-dependent and σ^{70}-dependent genes (Erickson *et al.*, 1987). Studies of the promoter region of *degP*, encoding a periplasmic protease, found similarity to the *rpoH* P3 promoter, and after σ^E was purified, it was demonstrated that *degP* transcription occurred in a σ^E –dependent manner (Lipinska *et al.*, 1989; Erickson *et al.*, 1989).

Three genes were discovered to code for proteins involved in σ^E signal transduction. *rpoE* codes for the sigma factor itself while *rseA* and *rseB* encode an inner membrane-bound anti-sigma factor and a periplasmic protein respectively (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997). The cytoplasmic domain of the RseA protein interacts with σ^E, sequestering it and therefore preventing σ^E-mediated transcription, while RseB interacts with the periplasmic domain of RseA (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997). The current model for σ^E signal transduction is that in the absence of envelope stress RseA binds σ^E, keeping it inactive while RseB stabilizes the RseA-σ^E complex (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997). Envelope stress causes misfolded outer membrane proteins that may titrate RseB away from RseA, allowing control of the degree of activation of the response (Raivio and Silhavy, 2001). This stress is believed to destabilize the interaction between RseA and σ^E, liberating the sigma factor and allowing expression of the σ^E regulon (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997). RseC is a minor positive regulator of σ^E with further roles in thiamine biosynthesis and the cellular oxidative stress response (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997; Beck *et al.*, 1997; Koo *et al.*, 2003). Conditions which activate the σ^E response have been shown to cause the sequential proteolytic degradation of RseA via the proteases DegS and YaeL, which would presumably cause the release of σ^E into the cytoplasm (Figure 1.1) (Ades *et al.*, 1999; Walsh *et al.*, 2003; Alba *et al.*, 2002; Kanehara *et al.*, 2001). DegS possesses a PDZ domain, which is found in a large variety of proteins and interacts with specific sequences in the C-terminus of a polypeptide (Walsh *et al.*, 2003). The PDZ domain of DegS has been demonstrated to be a negative regulator of protease activity and binds to the C-terminus of many outer membrane proteins (Walsh *et al.*, 2003). OMP-like peptides with YXF C-terminal domains, presumably exposed when OMPs are misfolded, interact with the PDZ domain of DegS, releasing inhibition of the protease domain (Walsh *et al.*, 2003). The DegS protease is then free to cleave the periplasmic domain of RseA, which catalyzes the cleavage of the cytoplasmic domain of RseA by YaeL. Proteolysis of RseA allows the release of σ^E, which can then interact with RNA polymerase and activate expression of the σ^E regulon. YaeL also has a PDZ domain, which, when deleted allowed YaeL to

Fig 1.1. The current model of σ^E signal transduction. Adapted from Alba and Gross (2004). In the absence of stress the PDZ-domain of the DegS protein inhibits protease activity and the cytoplasmic domain of RseA binds σ^E, sequestering it. Envelope stresses cause misfolding of outer membrane protein (OMP) periplasmic intermediates. The C-termini of these outer membrane proteins interacts with the PDZ-domain of DegS and alleviate inhibition of the DegS protease domain. The DegS protease cleaves the periplasmic domain of RseA, which catalyzes the cleaving of the cytoplasmic domain of RseA by YaeL. Proteolysis of RseA allows the release of σ^E, which can then interact with RNA polymerase and activate expression of the σ^E regulon.



Activation of σ^E regulon:
rseA, rseB, rseC, rpoE, degP, rpoH, fkpA, surA, skp, dsbC, others

cleave RseA independently of previous cleavage by DegS (Kanehara *et al.*, 2003). Deletion analysis of RseA revealed that a glutamine rich domain in the periplasm possesses a YaeL-inhibitory function (Kanehara *et al.*, 2003). This suggested that the initial cleavage of RseA by DegS removes inhibition of YaeL by either alleviating PDZ domain inhibition of YaeL or alleviating the repression of YaeL by the glutamine rich domain of RseA (Kanehara *et al.*, 2003).

1.1.1.1 The σ^E regulon

To date there have been twenty-two members of the regulon characterized. The expression of the *rseA*, *rseB* and *rseC* genes are all controlled by σ^E, and all three are believed to function as regulatory proteins in the transduction pathway (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997). As well, the sigma factor genes *rpoH* and *rpoE*, which encodes σ^E itself, are also controlled by the σ^E regulon (Rouviere *et al.*, 1995, Erickson *et al.*, 1987). Thus, envelope stress elevates levels of both the envelope stress and cytoplasmic heat shock sigma factors, which can then mediate the cellular transcriptional response. As previously mentioned, the periplasmic serine protease *degP* is controlled by the σ^E response, thus the misfolded proteins which occur as a result of envelope stressors such as heat or ethanol are degraded by DegP, alleviating the stress (Erickson *et al.*, 1989). Another member of the regulon is the periplasmic peptidyl-prolyl isomerase FkpA (Danese and Silhavy, 1997). Peptidyl-prolyl isomerases are enzymes localized in either the cytoplasm or periplasm that catalyze the interconversion of an X(aa)-Pro peptide bond between the *cis* and the *trans* states (Dartigalongue and Raina, 1998). Where DegP degrades misfolded proteins, FkpA attempts to refold the proteins properly.

Further members of the regulon were isolated using two independent genetic approaches. The cloning of small DNA fragments generated by genomic fragmentation into a single copy vector carrying a promoterless *lacZ* reporter and the random fusion of the *lacZ* gene to genomic regulatory regions via a λMu53-*lacZ* transposon were both used to create libraries (Dartigalongue *et al.*, 2001). These were then screened in a variety of backgrounds in order to isolate promoters that increased *lacZ* expression in a σ^E-dependent manner (Dartigalongue *et al.*, 2001). Twenty σ^E regulated promoters were isolated in this screen, and ten of these were previously identified σ^E regulon members (Daritgalongue *et al.*, 2001). In addition to σ^H and σ^E, the expression of the housekeeping sigma factor, σ^{70} was also upregulated (Daritgalongue *et al.*, 2001). σ^E regulated protein folding factors and degrading factors identified include *degP, fkpA*, the disulfide bond oxidoreductase *dsbC*, and the chaperones *skp* and *surA* (Dartigalongue *et al.*, 2001). The pathway also appears to increase the production of lipopolysaccharide as *rfaDFCL* and *lpxDA*, the products of which are known to be involved in the LPS biosynthetic pathway, are upregulated in a σ^E-dependent manner (Dartigalongue *et al.*, 2001). The products of the *ecfABC* operon are also predicted to be involved in LPS biogenesis (Dartigalongue *et al.*, 2001). Two members of the regulon involved in sensory functions are *mgoD*, which coordinates cellular pressure and synthesis of oligosaccharides in the periplasm, and *cutC*, which has been proposed to be involved in copper homeostasis (Dartigalongue *et al.*, 2001). The largest group of σ^E regulated promoters identified by Dartigalongue *et al.* (2001) encode inner membrane proteins (NlpE, EcfD, EcfG, EcfI, EcfL) or outer membrane proteins (EcfK and EcfM) of unknown function. EcfL and EcfE, later renamed YaeL, are members of the regulon which appear to be essential (Dartigalongue *et al.*, 2001).

The σ^E regulon was further expanded using a two-plasmid system. A library of chromosomal fragments was made upstream of a promoterless *lacZα* reporter and this library was introduced into a strain carrying an arabinose-inducible *rpoE* overexpression vector (Rezuchova *et al.*, 2003). This screen identified 11 promoters controlling 15 genes upregulated in a σ^E dependent manner. As with the screen performed by Dartigalongue *et al.* (2001) genes identified in the screen coded for factors involved in phospholipid and LPS biosynthesis (*psd, lpxP*), inner membrane proteins (*bacA* and *sbmA*) and outer membrane proteins (*smpA, yeaY*) (Rezuchova *et al.*, 2003). Other upregulated genes were the phosphohistidine phosphatase, *sixA*, and genes of an unknown function (*ybaB, yaiW, yiiS, yiiT, yfeY*) (Rezuchova *et al.*, 2003).

1.1.1.2 σ^E inducing cues

As mentioned, two major activating signals for the σ^E pathway are heat and ethanol, conditions which are known to denature proteins (Erickson *et al.*, 1987; Rouviere *et al.*, 1995). More specifically, it was found that decreasing the expression of outer membrane proteins reduced transcription of both an *rpoH3-lacZ* and a *degP-lacZ* reporter while overexpression of outer membrane proteins or introduction of mutations that cause misfolding in the periplasm resulted in increased reporter transcription (Mescas *et al.*, 1993). When proteins known to be misfolded in the periplasm were overexpressesed, the response was activated, while upregulation of outer membrane protein folding factors such as *fkpA* attenuated the activation (Missiakas *et al.*, 1996).

1.1.1.3 The role of the σ^E response

Specifically, the σ^E response appears to be geared towards sensing misfolded outer membrane proteins or the periplasmic accumulation of outer membrane protein intermediates (Mecsas *et al.*, 1993). Essentially, perturbations to the cell envelope that result in outer membrane protein misfolding result in the upregulation of protein degrading and folding factors to counter the damage. Based on its homology with a *Pseudomonas aeruginosa* protein, the σ^E response may play a role in *E. coli* pathogenesis. *Pseudomonas aeruginosa* contains a σ^E homologue, AlgU, responsible for the transcription of genes required to produce the exopolysaccharide alginate, which leads to a mucoid phenotype (Yu *et al.*, 1995). The AlgU transcriptional activator is controlled through an uncharacterized mechanism by an inner membrane bound anti-sigma factor, MucA, which is homologous to RseA (Xie *et al.*, 1996; Schurr *et al.*, 1996). The MucA-AlgU interaction appears to be stabilized by a periplasmic protein, MucB, that like RseB, interacts with the membrane bound anti-sigma factor (Martin *et al.*, 1993; Schurr *et al.*, 1996). The conversion of cells to this mucoid phenotype is believed to be a key step in *P. aeruginosa* pathogenicity with respect to lung infections of cystic fibrosis patients (Govan and Deretic, 1996).

1.2 The Cpx Two-Component Signal Transduction System

The Cpx two-component system was originally identified by the effect *cpxA* mutations had on conjugal transfer of the F plasmid as a result of a non-functional F pilus (McEwen and Silverman, 1980a). These mutations had pleiotropic effects which included temperature sensitivity, leucine and valine auxotrophy, and altered membrane protein profiles (McEwen and Silverman, 1980b; McEwen and Silverman, 1980c; McEwen and Silverman, 1982). The majority of these phenotypes result from disruption of envelope associated processes (Raivio and Silhavy, 2001). Subsequent characterization demonstrated that the Cpx pathway is controlled by a typical two-component system comprised of a membrane bound sensor kinase CpxA, and a cytoplasmic response regulator, CpxR (Fig.1.2) (Weber and Silverman, 1988; Dong *et al.*, 1993). The CpxA protein is a 52 kDa polypeptide consisting of 457 amino acids localized to the inner membrane, and contains two hydrophobic transmembrane domains along with the conserved histidine kinase domain in the cytoplasmic portion of the protein (Weber and Silverman, 1988). A domain that responds to envelope stress was identified in the periplasmic region of CpxA (Raivio and Silhavy, 1997). In the presence of an activating cue the conserved histidine residue of CpxA is autophosphorylated (Hoch and Silhavy, 1995; Raivio and Silhavy, 1997). The phosphorylated histidine can then serve as a substrate for the phosphorylation of the response regulator, CpxR, at a conserved aspartate residue, which is mediated by a domain on the response regulator (Hoch and Silhavy, 1995). This allows CpxR to serve as a transcriptional activator (Hoch and Silhavy, 1995). Like many other histidine kinases, CpxA also contains a conserved domain which functions as an aspartyl-phosphate phosphatase, thus dephosphorylating the response regulator in the absence of an activating signal (Raivio and Silhavy, 1997). The cognate response regulator of CpxA, CpxR, is a 26.2 kDa polypeptide consisting of 232 amino acids (Dong *et al.*, 1993). The C-terminal domain of the CpxR protein bears homology to the OmpR subclass of response regulators, which places it in the winged helix class of transcriptional activators (Martinez-Hackert and Stock, 1997). The presence of the low molecular weight molecule acetyl phosphate can also phosphorylate CpxR, enhancing its efficiency as a transcriptional activator (Pogliano *et al.*, 1997). In a CpxA null strain, growth conditions which diminish cellular acetyl phosphate prevent CpxR from activating transcription of downstream target genes (Danese *et al.*, 1995). Growth conditions that enhance levels of acetyl-phosphate permit CpxA-independent phosphorylation of CpxR and subsequent activation of downstream targets (Danese *et al.* 1995).

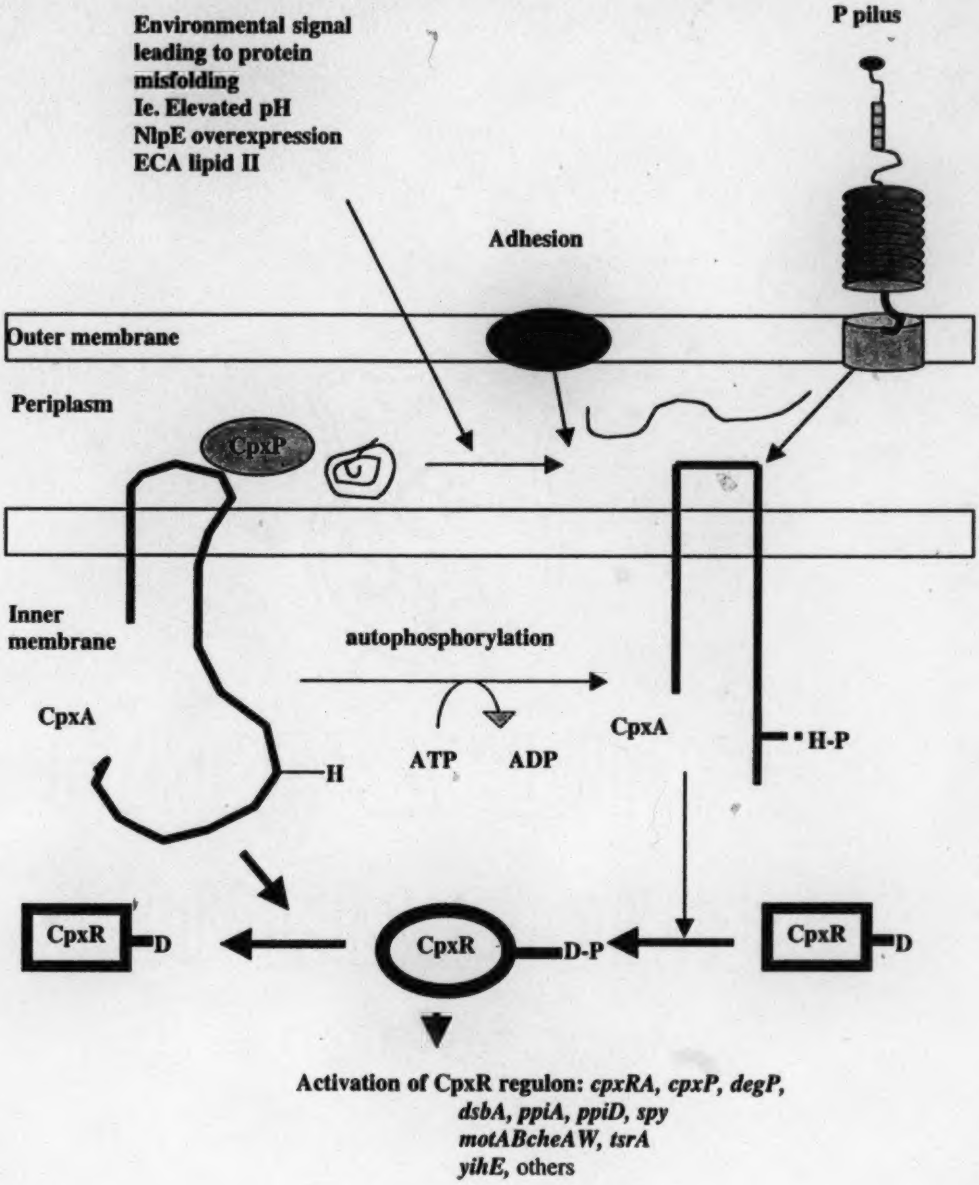
1.2.1 The Cpx Regulon

Until recently, the Cpx regulon consisted of a group of genes which could be divided into four categories: protein folding factors, protein degrading factors, chemotactic/motility proteins, and components of the Cpx pathway (for a review see Raivio and Silhavy, 2001, Raivio *et al.*, 2000). Under Cpx inducing conditions, which will be discussed in section 1.2.2, the products of the upregulated regulon genes work in concert to remove damaged periplasmic proteins and fine tune the stress response. A recent bioinformatics approach involving a genomic search for consensus CpxR-P binding sequences identified numerous new members of the regulon (Section 1.2.1.5), the roles of which are not completely understood.

1.2.1.1 Protein Folding Factors

The Cpx two-component system upregulates the expression of three protein folding factors in response to envelope stress and these factors fall into two general classes, disulfide bond oxidoreductases and peptidyl-prolyl isomerases (Danese and

Fig 1.2. The current model of the Cpx two-component signal transduction system. Adapted from DiGiuseppe and Silhavy (2003). CpxP prevents CpxA from being autophosphorylated, but in the presence of misfolded envelope proteins the CpxP mediated inhibition is relieved. The Cpx pathway can also be activated by adhesion to a hydrophobic surface, a signal which is NlpE dependent, or by misfolded P pilus subunits in the periplasm. CpxA, when activated, autophosphorylates at a conserved histidine residue and proceeds to phosphorylate CpxR. CpxR-P can then activate the transcription of genes controlled by the Cpx pathway. In the absence of an activating signal CpxA acts as a phosphatase, dephosphorylating CpxR.


Environmental signal
leading to protein
misfolding
Ie. Elevated pH
NlpE overexpression
ECA lipid II
P pilus
Adhesion
Outer membrane
Periplasm
CpxP
Inner
membrane
CpxA
H
autophosphorylation
ATP
ADP
CpxA
H-P
CpxR
D
CpxR
D-P
CpxR
D
Activation of CpxR regulon: *cpxRA, cpxP, degP,*
dsbA, ppiA, ppiD, spy
motABcheAW, tsrA
yihE, others

Silhavy, 1997; Pogliano *et al.*, 1997; Dartigalongue and Raina, 1998; Raivio and Silhavy, 2000). The gene *dsbA* codes for a disulfide bond oxidoreductase, an enzyme that is responsible for forming disulfide bonds between cysteine residues in the oxidizing environment of the *E. coli* periplasm (Rietsch and Beckwith, 1998). *dsbA* is located in an operon downstream of *yihE*, a gene of unknown function. Transcriptional fusion studies using a *yihE-dsbA-lacZ* fusion revealed that in the presence of *nlpE* overexpression, a Cpx activating cue, there was a 5.3 fold increase in reporter activity while there was negligible transcription of the fusion in a *cpxR*$^-$ or *cpxA*$^-$ background (Danese and Silhavy, 1997). Studies of the *dsbA* sequence elucidated that Cpx-controlled transcription is initiated at a distal promoter upstream of *yihE* (Pogliano *et al.*, 1997). In *Salmonella enterica* serovar Typhimurium, recent evidence has demonstrated that while an absence of *yihE* (renamed *rdoA*) affected the Cpx-dependent activation of *dsbA*, a bicistronic transcript was not involved in regulation as the Cpx-dependent transcription of *dsbA* could be restored to wild type levels by providing *rdoA* to the mutant in *trans* (Suntharalingham *et al.*, 2003). Further study demonstrated that Cpx activation leads to increases in both *rdoA* and *dsbA* transcript (Suntharalingham *et al.*, 2003). Therefore, in *E. coli* and *S. enterica* sserovar Typhimurium, Cpx activation leads to increased levels of the disfulfide oxidase DsbA and the gene upstream of *dsbA*, *yihE*. The gene *ppiA* encodes a peptidyl-prolyl isomerase (ppiase) in the cyclophilin class, so named for its affinity for the drug cyclosporine (Liu and Walsh, 1990). DNaseI footprinting studies identified a CpxR binding site in the promoter region of *ppiA*, while *nlpE* overexpression increased expression from the *ppiA* promoter 2.5 fold (Pogliano *et al.*, 1997). This effect was abolished in strains containing a *cpxRA* deletion (Pogliano *et al.*, 1997). The gene *ppiD* codes for a second ppiase regulated by the Cpx system and is grouped in a class of folding factors resembling the *E. coli* ppiase parvulin (Dartigalongue and Raina, 1998). The *ppiD* gene was identified based on the ability of multiple copies to suppress a *surA* mutation which usually results in misfolded envelope proteins (Dartigalongue and Raina, 1998). A *ppiD-lacZ* fusion in a *cpxR* null background was expressed at lower levels than in a wild type background, identifying *ppiD* as a Cpx regulated gene (Dartigalongue and Raina, 1998). However, it was never demonstrated that Cpx-activating signals resulted in increased *ppiD* expression. Interestingly the *ppiD* locus is also under the control of σ^H, the major cytoplasmic heat-shock factor (Dartigalongue and Raina, 1998).

1.2.1.2 Protein Degrading Factors

Envelope proteins which cannot be repaired by protein folding factors must be degraded by periplasmic proteases. The serine endoprotease DegP was first identified through a mutation that prevented cells from degrading misfolded periplasmic proteins (Strauch and Beckwith, 1998). Subsequent studies found gain-of-function mutations that increased DegP activity and protected the cell from toxic envelope proteins. These mutations mapped to the *cpx* locus (Cosma *et al.*, 1995). S1 nuclease protection assays and *degP-lacZ* fusion activity in Cpx-activated backgrounds demonstrated that *degP* was activated by the Cpx response (Danese *et al.*, 1995). As mentioned previously, *degP* expression is also increased in heat-shock conditions, an effect mediated through the σ^E pathway independent of the Cpx system (Danese and Silhavy, 1995). Deletion analysis revealed that DegP has chaperone activity and this activity is functionally redundant with SurA, a member of the σ^E regulon (Rizzitello *et al.*, 2001).

1.2.1.3 Autoregulation of the Cpx System

In addition to folding and degrading factors, the CpxR regulon also includes the *cpxRA* (Raivio *et al.*, 1999) genes as well as the *cpxP* gene (Danese and Silhavy, 1998). Early analysis of the *cpxRA* sequence identified a putative CpxR binding motif of 5'-GTAAA(N5)GTAAA-3' upstream of the *cpxRA* operon, suggesting that the Cpx system may control its own expression (Pogliano *et al.*, 1997). Subsequent immunoblot studies then demonstrated that, in a gain of function CpxA* mutant, the levels of CpxA and CpxR proteins increase in comparison to a wild-type background, indicating autoactivation (Raivio *et al.*, 1999). A *cpxR-lacZ* fusion showed a three to five fold increase in transcription using any of three *cpxA** gain of function alleles, while the presence of either a *cpxA* or *cpxR* knockout reduced transcription of the fusion (Raivio *et al.*, 1999). As well, overexpression of *nlpE*, a known Cpx activating cue, increased the transcription of the *cpxR-lacZ* fusion threefold in a CpxR-dependent manner (Raivio *et al.*, 1999). Thus, under inducing conditions, a positive feedback system exists in which activation of the Cpx system increases its expression, allowing for a more intense response.

A screen using a transposon that created random promoterless *lac* operon fusions throughout the *E. coli* chromosome identified *cpxP*, a gene that demonstrated increased transcription in the presence of *nlpE* overexpression and was immediately upstream of *cpxR* (Danese and Silhavy, 1997). The *cpxP* locus is divergently expressed from *cpxR* and is regulated in *trans*, as a *cpxP-lacZ* fusion inserted at the λ*att* site displayed the same increase in transcription as the fusion generated by the screen (Danese and Silhavy, 1997). The fusion of the first 71 codons of the *cpxP* ORF to the alkaline phosphatase

(AP) coding sequence showed that CpxP is a periplasmic protein, since AP activity, which requires the oxidizing environment found in the periplasm, was observed (Danese and Silhavy, 1997). Overexpression of *cpxP* from an inducible *trc* promoter led to reduced transcription of a chromosomal *cpxP-lacZ* fusion in a CpxA-dependent manner indicating that CpxP downregulates the Cpx regulon via CpxA (Figure 1.2, Raivio *et al.*, 1999). CpxP expression decreased levels of the *cpxP-lacZ* fusion reproducibly in a strain carrying a *cpxA** mutation in the cytoplasmic region of the protein which leaves the periplasmic sensing domain intact (Raivio *et al.*, 1999). Conversely, in a CpxA* mutant containing a periplasmic domain mutation that results in perpetual maximal activity, a phenotype referred to as "signal blind", overexpression of CpxP had no effect on the *cpxP-lacZ* fusion. Thus, CpxP requires the periplasmic sensing domain of CpxA to repress activation of the regulon (Raivio *et al.*, 1999). Finally, it was shown that spheroplast formation activates the Cpx pathway, possibly by titrating CpxP away from CpxA. However, the tethering of a MBP-CpxP fusion protein to the inner membrane via a signal sequence mutation prevents induction of the Cpx regulon by spheroplasting (Raivio *et al.*, 2000). This suggests that some sort of interaction between CpxP and CpxA is occurring to mediate inhibition of the pathway (Figure 1.2, Raivio *et al.*, 2000). The precise nature of this interaction remains unknown, but it is thought that envelope stress either causes misfolding of CpxP itself or misfolding of periplasmic proteins that CpxP then binds to (Raivio *et al.*, 2000). Once CpxP is removed from CpxA, the stress response can then be activated (Figure 1.2).

Spheroplast induction was also previously demonstrated to induce expression of a novel periplasmic protein, Spy (Hagenmaler *et al.*, 1997). A *spy-lacZ* transcriptional fusion increased in expression like other members of the Cpx regulon when placed in a strain containing a *cpxA** mutation or when *nlpE* was overproduced. The function of Spy remains unknown and it was placed in this section because the only known homologue of Spy is CpxP (Raivio *et al.*, 2000).

1.2.1.4 Chemotactic/Motility Factors

The identification of a CpxR-P binding sequence through electrophoretic mobility shift assays and DnaseI protection assays of the *dsbA, degP,* and *ppiA* promoters lead to a screen of *E. coli* promoters for the presence of CpxR consensus binding sites (Pogliano *et al.*, 1997). This screen identified two putative, negatively regulated, new members of the Cpx regulon involved in motility and chemotaxis (DeWulf *et al.* ,1999). These genes are the first members of the regulon shown to be repressed by CpxR-P. A perfect CpxR binding sequence was found overlapping the -35 region of the *motABcheAW* operon, which encodes flagellar motor proteins and proteins involved in the chemotaxis signal transduction pathway (DeWulf *et al.*, 1999). Electrophoretic mobility shift assays subsequently verified the interaction between CpxR and the *motABcheAW* promoter while Northern analysis supported the negative regulation of the operon by CpxR-P (DeWulf *et al.*, 1999). However, Northern analysis and swarm assays performed in a *cpxR* null mutant showed that, while levels of *motABcheAW* mRNA remained unchanged, the swarming activity was notably higher, indicating that additional regulatory factors exist (DeWulf *et al.*, 1999). The *tsr* gene, encoding a serine chemoreceptor that functions as a methyl accepting chemotaxis protein, was shown to have severely attenuated expression in a CpxA* strain, indicating that it is also a negatively regulated member of the Cpx regulon (DeWulf *et al.*, 1999). While the experimental evidence demonstrates that the aforementioned genes are part of the Cpx regulon, it does not address the question of whether activation of the wildtype Cpx pathway inhibits motility and chemotaxis.

1.2.1.5 Putative CpxR regulon members isolated through bioinformatics

DeWulf *et al.* (2002) extended the bioinformatics approach used in their earlier study by comparing a weighted matrix representing the CpxR-P binding site to 15 base sections of the genome. Statistical analysis was used to group putative CpxR-P binding sites according to the degree they matched the CpxR-P consensus binding sequence derived from 10 Cpx regulated promoters (DeWulf *et al.*, 2002). The screen identified around 100 putative operons under Cpx control and eight of these operons were further characterized, demonstrating CpxR-dependent regulation by Northern analysis (DeWulf *et al.*, 2002). Identified genes upregulated by CpxR include *ung*, a uracil-N-glycosylase, *ompC*, the outer membrane protein which, when overexpressed, activates the σ^E pathway, *psd*, a phosphatidyl serine decarboxylase, *mviM*, a virulence factor, *aroK*, a shikimate kinase, and *secA*, the ATPase of the general secretory pathway, (DeWulf *et al.*, 2002). Genes demonstrated to be downregulated in a CpxR-dependent manner included *rpoErseABC*, the operon encoding σ^E and its regulatory proteins, and *aer*, an aerotaxis sensor protein (DeWulf *et al.*, 2002).

1.2.2 Cpx activating cues

The first known activating cue of the Cpx pathway was overexpression of the *nlpE* gene. The NlpE protein was identified as a suppressor of the toxic activity of periplasmic LacZ and was localized to the outer membrane (Snyder *et al.*, 1995). For years *nlpE* overexpression was used to activate the Cpx pathway with no knowledge of the NlpE protein function. Recent evidence has implicated NlpE in transducing an adhesion signal to the Cpx two-component system (Otto and Silhavy, 2002). Bead binding assays, in which bacteria containing *lacZ* fusions to various Cpx regulated genes were allowed to adhere to hydrophobic beads, revealed that the Cpx envelope stress response is activated by attachment to hydrophobic surfaces. Further, activation is mediated by NlpE since *nlpE* null mutants had similar fusion activity to *cpxR* and *cpxA* null mutants (Otto and Silhavy, 2002). As well, quartz crystal microbalance studies, which measure the dynamic interactions of the adhered bacteria with a crystal surface, indicated that in addition to having identical attachment dynamics, *nlpE, cpxR,* and *cpxA* mutants all had decreased adhesion but increased cell surface contact relative to wildtype bacteria (Otto and Silhavy, 2002). The work by Otto and Silhavy (2002) implicated both NlpE and CpxAR in bacterial adherence.

While the Cpx activating adhesion signal is NlpE-dependent, other Cpx activating cues, such as elevated pH, appear to be NlpE independent. Studies of *Shigella sonnei virF* expression in *E. coli* demonstrated a Cpx-dependent increase in expression of *virF*, a positive regulator of virulence genes, at pH 7.4, whereas *virF* was repressed at pH 6.0 (Nakayama and Watanabe, 1995). No *virF* transcript accumulation was seen in *cpxR* null strains of *S. sonnei* at either pH 7.4 or 6.0, suggesting that CpxR is an essential activator of *virF* (Nakayama and Watanabe, 1995). Experiments performed by Danese and Silhavy (1998) in *E. coli* showed that a *cpxP-lacZ* fusion had a 50-fold increase in activity when the cells were grown in pH 8.4 as opposed to pH 5.3, an effect that was abolished in a *cpxA* null strain. As well, when *cpxRA* and *cpxP* mutant growth was compared to wildtype cell growth over a pH range from 7.0 to 9.4 the mutants displayed hypersensitivity to alkaline conditions as they failed to grow between pH 8.8 and 9.4 (Danese and Silhavy, 1998). Thus, the Cpx response is also activated by, and important for survival at, elevated pH.

Two types of alterations to the inner membrane are also known to activate the Cpx pathway. *E. coli* mutants for the enzyme phosphatidylserine synthase completely lack phosphatidylethanolamine and this absence was shown to be a potent activator of the Cpx pathway (Mileykovskaya and Dowhan, 1997). As phosphatidylethanolamine is required for the proper folding of some membrane proteins, the activation of the Cpx pathway in the null mutant may reflect misfolded membrane proteins (Bogdanov and Dowhan, 1998). Mutations in the biochemical pathway of enterobacterial common antigen (ECA) that cause the intermediate ECA lipid II to accumulate in the inner membrane activate both the Cpx and σ^E pathways (Danese *et al.*, 1998). It was noted that these mutants had phenotypes associated with misfolded envelope proteins and this may be the activating cue in ECA lipid II accumulation (Danese *et al.*, 1998). Finally, exposure to EDTA has recently been shown to cause Cpx activation, potentially by increasing the permeability of the outer membrane (Nikaido, 1996). The final known Cpx activating cue is aggregation of misfolded chaperone-usher type pilus subunits in the periplasmic space, a subject which will be discussed in section 1.3 (Hung *et al.*, 2000).

1.3 Role of the Cpx Pathway in Pilus Biogenesis

1.3.1 Pili and Uropathogenic *Escherichia coli*

Uropathogenic *E. coli* (UPEC) are the primary cause of urinary tract infections, and can cause bladder infections and pyelonephritis if the bacteria are able to ascend the urinary tract (Hung and Hultgren, 1998; Schilling *et al.*, 2001). UPEC expresses two types of pili which are important to its pathogenicity: type I pili and P pili (Hung and Hultgren, 1998). Pili are long filamentous protein structures which contain adhesins on the tip that mediate binding to host receptors (Hultgren *et al.*, 1996) The type I pilus is essential for bladder infections whereas the P pilus is crucial for mediating attachment to kidney epithelial cells in pyelonephritis (Roberts *et al.*, 1994; Hung and Hultgren, 1998). In order to understand the role of the Cpx system in pilus biogenesis one must first understand the conserved mechanism whereby P and type I pili are assembled, the chaperone usher pathway.

1.3.2 The Chaperone Usher Pathway

Correct assembly of chaperone-usher type pili in Gram negative bacteria requires that the pilus components, which are translated in the cytoplasm, cross the inner membrane, fold properly in the periplasm, reach the proper location in the outer membrane and are correctly added on to the growing pilus structure (for a recent review see Thanassi and Hultgren, 2000). As it is extremely well characterized, the assembly of P pili will be used in this section to explain the processes involved in the chaperone usher pathway.

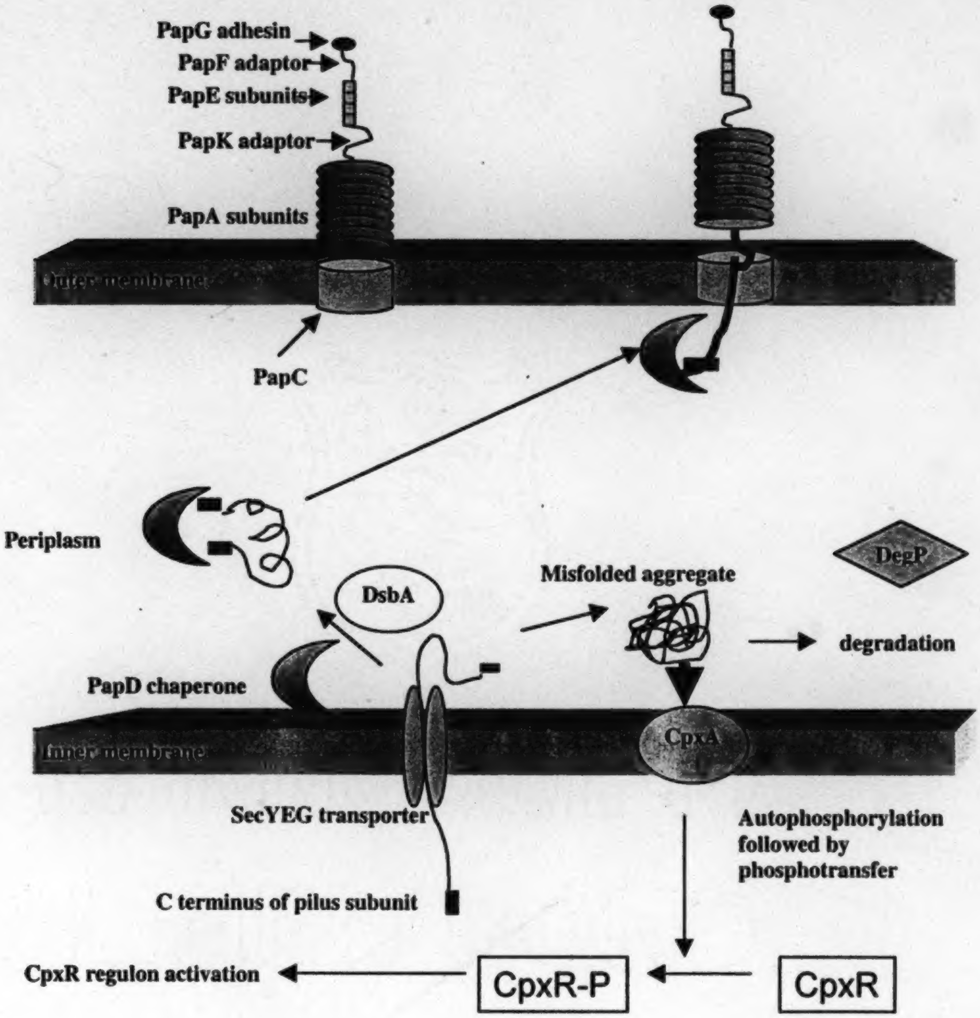
The P pilus is coded for by the *pap* gene cluster. The cluster encodes 11 genes, two of which have a regulatory function, six are structural components, one is an usher and one is a chaperone (for a review see Thanassi and Hultgren, 2000). The pilus is comprised of a thick rod and then a tip fibrillum which is distal to the bacterial cell surface (Fig 1.3). The fibrillum of the pilus is comprised of PapG, an adhesin that binds to Galα(1–4)Gal moieties on kidney epithelial glycolipids, PapF, an adaptor which joins PapG to PapE, PapE which is the major tip fibrillum component, and PapK which binds the pilus tip to the pilus rod (Bock *et al.*, 1985; Kuehn *et al.*, 1992). The pilus rod is comprised of PapA, the major subunit, which is terminated and potentially anchored to the outer membrane by PapH (Båga *et al.*, 1984; Båga *et al.*, 1987). PapD is a periplasmic chaperone while PapC is the outer membrane bound usher (for a review see Hung and Hultgren, 1998).

Pilus subunits translated in the cytoplasm are transported into the periplasm using the SecYEG transporter of the general secretory pathway and their signal sequence is then cleaved (Thanassi and Hulgtren, 2000). Interaction with the Cpx-regulated folding factor DsbA ensures proper disulfide bonds are formed in the PapD chaperone and the PapG subunit (Jacob-Dubuisson *et al.,* 1994). In the absence of DsbA, PapD cannot fold into its native conformation and subsequently is unable to interact with any of the Pap subunits (Jacob-Dubuisson *et al.,* 1994). Interestingly, in the absence of PapD, disulfide bonds can still be formed by DsbA in PapG, suggesting that perhaps DsbA binds the subunit as it passes through the transporter then passes it on to PapD (Jacob-Dubuisson *et al.*, 1994). The PapD chaperone has three functions: i) it binds to pilus subunits, capping the active sites and preventing the subunits from forming non-productive aggregates; ii) it functions to help transport the pilus subunits into the periplasm; and iii) along with DsbA it ensures proper folding of the subunits (for a review see Sauer *et al.*, 1999). The PapD-subunit complex is targeted to the outer membrane-bound PapC usher where the chaperone dissociates from the pilus subunit (Thanassi *et al.*, 1998). The subunits pass in unwound form through the oligomeric channel formed by multiple PapC proteins (Thanassi *et al.*, 1998). Deletion analysis has shown that the N-terminus of PapC is targeted by the chaperone-subunit complex and that the C-terminus plays an unknown role in biogenesis of the pilus (Thanassi *et al.*, 2002). In UPEC type I pili, also assembled through the chaperone-usher pathway, the adhesin, FimH was demonstrated to target the chaperone-subunit complex to the usher (Barnhart *et al.*, 2003). A model is shown in Figure 1.3.

1.3.3 Phase variation of P pili

The expression of P pili is regulated in that bacteria can undergo phase variation in which pili are expressed (phase ON) or absent (phase OFF) (Blyn *et al.*, 1989). Pilus expression is important in pathogenesis but the pili are highly immunogenic, thus expression must be tightly attuned to the environment so the bacteria can either express the pili when needed or repress them under inappropriate conditions (van der Woude *et al.*, 1996). P pilus phase variation was found to be mediated by the binding of numerous regulatory factors within the promoter region. There are two divergent promoters in the *pap* operon, the *papI* and *papBA* promoters (Hung and Hultgren, 1998). The *papI* gene encodes a regulatory protein that is responsible for the OFF-ON phase variation of the *papBA* promoter that controls the expression of the pilus structural genes

Fig 1.3. A model of the role of the Cpx two-component signal transduction system in the chaperone-usher pathway of P pilus biogenesis. Adapted from Thanassi and Hultgren, 2000. SecYEG transports the pilus subunits across the inner membrane where DsbA and PapD correctly fold the subunit. PapD escorts the pilus to the usher, PapC, which adds the subunit onto the growing pilus. In the absence of DsbA-chaperone interaction, misfolded subunit aggregates form, inducing the autophosphorylation of CpxA, which leads to the phosphorylation of CpxR. Activation of the CpxR regulon increases *dsbA* expression, which helps correctly fold the subunits and also increases the level of DegP, which degrades the misfolded aggregates.

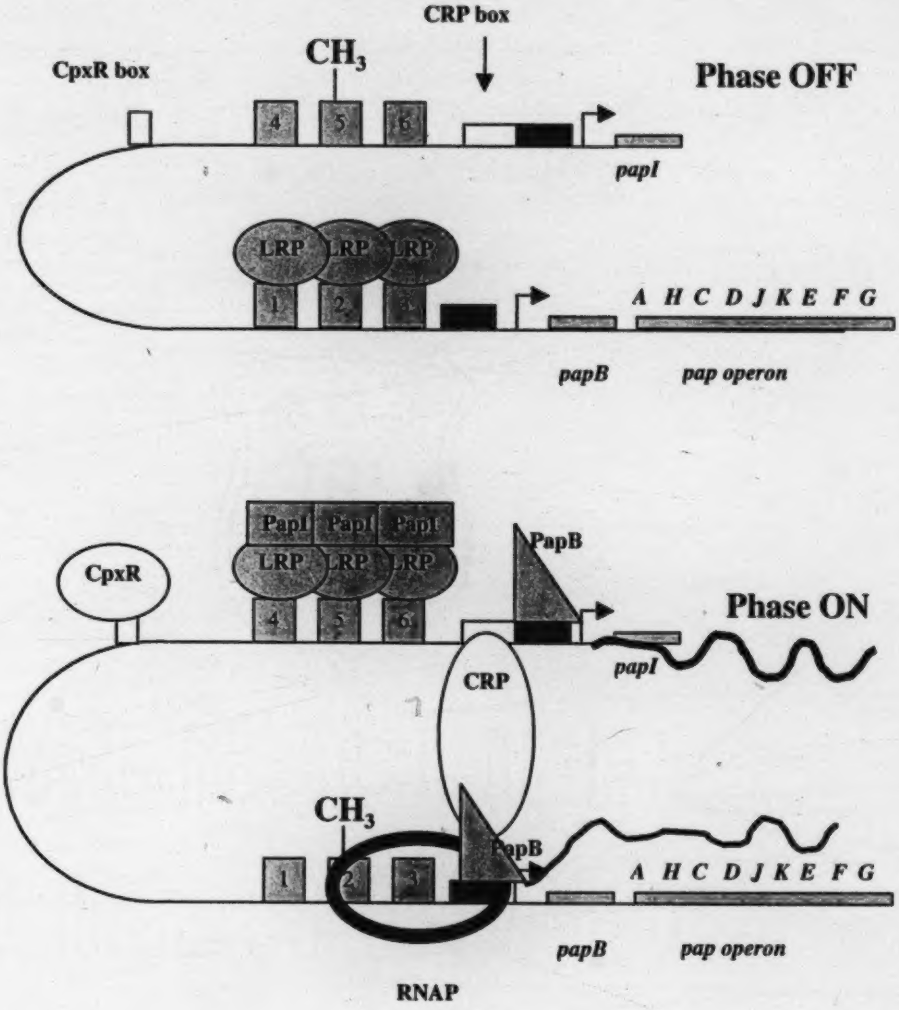

PapG adhesin
PapF adaptor
PapE subunits
PapK adaptor
PapA subunits
Outer membrane
PapC
Periplasm
DegP
Misfolded aggregate
DsbA
degradation
PapD chaperone
Inner membrane
CpxA
SecYEG transporter
Autophosphorylation followed by phosphotransfer
C terminus of pilus subunit
CpxR regulon activation
CpxR-P
CpxR

(Kaltenbach *et al.*, 1995; see Fig. 1.4). The catabolite activator protein (CRP) and PapB can bind to sites upstream of the *papI* promoter to increase its transcription (Forsman *et al.*, 1989, 1992; see Fig. 1.4). CRP is required to interact with the α-subunit of RNA polymerase (RNAP), as mutation within the RNAP interacting domain of the CRP protein eliminated transcription of the operon (Weyland *et al.*, 2001). LRP (leucine responsive protein) is a transcription factor essential for the expression of the *pap* operon. The promoter regions of *papBA* and *papI* contain two DAM methylation sites, known as GATC-I and GATC-II, only one of which is ever methylated, as well as six LRP binding sites which overlap the two methylation sites (van der Woude *et al.*, 1996). The methylation state of GATC-I and -II are responsible for the transcriptional regulation of the *pap* operon, as LRP displays a two fold lower affinity for methylated binding sites (Hale *et al.*, 1998). When the first three LRP binding sites are filled, the methylation of GATC-II is blocked, therefore only GATC-I is methylated and the *pap* operon is phase OFF (van der Woude *et al.*, 1996). When PapI is present it interacts with LRP to drive the movement of LRP to binding sites 4, 5 and 6, blocking methylation of GATC-I (van der Woude *et al.*, 1995). This results in methylation of GATC-II, which allows CRP binding to the promoter region and recruitment of RNA polymerase, allowing expression of the *pap* operon (van der Woude *et al.*, 1995).

1.3.4 The Cpx pathway and P pilus assembly

When the expression of plasmid-encoded P pili from the natural promoter in a *cpxR* null strain was studied, it was observed using transmission electron microscopy that

Fig 1.4: Phase variation of P pili in uropathogenic *Escherichia coli*. Adapted from Hung *et al.*, 2001. In the absence of PapI, LRP will bind to boxes 1, 2, and 3, preventing GATC-II methylation, and forcing GATC-I to be methylated. This prevents transcription of the *pap* operon. When PapI is present it binds to LRP, driving its translocation to boxes 4, 5, and 6, and preventing GATC-I methylation. CRP and PapB can now bind to boxes upstream of the *papI* promoter, enhancing transcription. CRP helps RNAP load onto the *pap* operon promoter and transcription proceeds.



▨ **1-6 denote LRP boxes. Box 2 is GATC-II. Box 5 is GATC-I**

■ **Denotes Pap B boxes**

the pili produced were shorter than in the wildtype (Hung *et al.*, 2000). When the pili number was examined using $MgCl_2$ precipitation, which relies on specific cross-linking of the PapA rods, the *cpxR* null strain expressed five fold fewer PapA subunits, reflecting that the strains were producing fewer, shorter pili (Hung *et al.*, 2000). When the *pap* operon was cloned behind an inducible *trc* promoter and overexpressed in a *cpxR* null strain, the same shortened pili were observed (Hung *et al.*, 2000). This suggests that the Cpx pathway controls factors required to assemble pili and that the mutant phenotype is independent of any Cpx regulation of transcription of the *pap* operon (Hung *et al.*, 2000). Indeed, in the absence of the chaperone PapD, the pilus subunits are said to go OFF-pathway and form aggregates which are subsequently degraded by the Cpx-regulated periplasmic protease DegP (Jones *et al.*, 1997). Even in the presence of the chaperone, a certain proportion of the pilus subunits fail to come into contact with the chaperone and are also driven OFF pathway (Hung *et al.*, 1999). As previously mentioned, the product of the Cpx regulated gene *dsbA* is essential for proper folding of both PapG and PapD, the chaperone, which is required to prevent the other subunits from aggregating and escort them to the usher (Jacob-Dubuisson *et al.*, 1994). Therefore Cpx-regulated factors are required for the assembly of P pili, an envelope-associated virulence determinant.

The *cpxR* null cells, which express P pili from the natural promoter, in addition to having shorter pili, also showed an increase in non-piliated cells, suggesting that the Cpx system plays a role in phase variation (Hung *et al.*, 2000). Studies of the sequence between the *papI* and *pap* operon promoters found several putative CpxR binding sequences (Hung *et al.*, 2000). Electrophoretic mobility shift assays showed that the sites were CpxR binding sites, and CpxR could only bind in the phosphorylated form (Hung *et* *al.*, 2000). Cells grown in glucose are in phase OFF as both the *papI* and *papBA* promoters are under catabolite repression (Baga *et al.*, 1985; Forsman *et al.*, 1992). However, it was found that *nlpE* overexpression, a potent Cpx activating cue, was able to overcome the catabolite repression and increase transcription from the *pap* operon (Hung *et al.*, 2000). Together, these data suggest that the Cpx response also regulates P pili at a transcriptional level.

1.3.5 The Cpx System and bacterial pathogenesis

All the initial research on the Cpx system was performed using lab strains of *E. coli* as the model organism. However, the observation that the Cpx two-component signal transduction system is essential for proper P pilus biogenesis (an essential UPEC virulence factor) gave rise to the hypothesis that the Cpx system is involved in proper assembly of envelope associated virulence determinants in pathogenic organisms (Raivio and Silhavy, 2000). As mentioned, studies of the DNA sequence upstream of virulence genes located on a UPEC pathogenicity island have identified putative CpxR binding sites (Hung *et al.*, 2000). This raises the intriguing possibility that upregulation of the Cpx response by P pilus assembly could subsequently lead to expression of virulence determinants required for mediating infection after adhesion (Raivio, 2001). Indeed, studies in other organisms have shown that Cpx-regulated genes or their homologues are involved in the assembly of envelope-associated virulence determinants.

1.3.5.1 DsbA and bacterial virulence determinants

The type IV pili are long, polar appendages involved in twitching motility, aggregation and adhesion and are a major virulence determinant in many bacteria such as *Vibrio cholerae* and enteropathogenic *E. coli* (EPEC) (Peek and Taylor, 1992; Giron *et al.*, 1991). Type IV pili are assembled independently of the chaperone-usher pathway in a manner resembling *sec*-dependent type II protein secretion (Bitter *et al.*, 1998). Export of the pilus subunit requires SecYEG, the protein complex which is required for transport of type II secreted proteins across the membrane (Thanassi and Hultgren, 2000). Both the toxin co-regulated pilus in *V. cholerae* and the bundle-forming pili (BFP) in EPEC require either DsbA or its homologue, TcpG, to create a mature pilus by the formation of a disulfide bond (Peek and Taylor, 1992; Zhang and Donnenberg, 1996). A DsbA homologue is required for the proper formation of proteases and multi-drug resistance systems in the pathogen *Burkholderia cepacia* (Hayashi *et al.*, 2000). As well, when a DsbA homologue was mutated in the phytopathogen *Erwinia cartovara*, there was reduced secretion of the virulence determinant pectate lyase and decreased maceration of plant tissues (Vincent-Sealy *et al.*, 1999). The pathogen *Yersinia pestis* requires the action of the DsbA protein for correct folding of an outer membrane protein involved in the type III secretion of Yop effector proteins into host cells (Jackson and Plano, 1999). Finally, the invasive pathogen *Shigella flexneri* also requires a DsbA homologue to properly fold a protein that helps form the type III secretory apparatus required for invasion of host cells (Yu, 2000; Yu *et al.*, 1998). These observations are particularly intriguing as EPEC also utilizes a type III secretory apparatus to modify host epithelial cells during infection.

1.3.5.2 DegP and bacterial virulence determinants

Expression of the serine protease DegP, which functions to degrade misfolded proteins, is controlled by both the σ^E and Cpx signal transduction pathways. Homologues of DegP, which are also known as HtrA, are required for full pathogencity in a number of bacteria. The absence of HtrA in the intracellular pathogen *Brucella abortis*, which causes undulate fever in humans, results in decreased phagocytic killing (Phillips and Roop II, 2001). In *Streptococcus pyogenes* a *degP* null mutation resulted in the need for a 35-fold higher number of bacteria to kill half the test mice inoculated in comparison to the wild type strain, evidence that *degP* is a critical virulence factor for this organism (Jones *et al.*, 2001). A *Y. pestis* strain with a mutant *htrA* gene showed a small increase in sensitivity to oxidative stress, had attenuated growth in an animal model, but most interestingly displayed a different protein expression profile at 28°C, which is the temperature of its flea vector (Williams *et al.*, 2000). A transposon mutagenesis screen designed to create avirulent *Salmonella typhimurium* mutants identified a mutant with an insertion in a gene homologous to *htrA* that exhibited impaired ability to survive and replicate inside host tissues (Johnson *et al.*, 1991). As well, the mutants displayed an increased susceptibility to oxidative stress suggesting a reduced ability to withstand oxidative killing in phagocytic cells (Johnson *et al.*, 1999). Indeed, evidence obtained in a phase II clinical vaccine trial of a *Salmonella enterica* serovar Typhi strain attenuated through the deletion of the *htrA* locus further supported development of the vaccine. Antibody response to the *htrA* mutant was high, but the organism failed to enter the blood, a characteristic of *S. enterica* serovar Typhi infection (Tacket *et al.*, 1999). The emerging pathogen *Bartonella henselae*, responsible for cat scratch disease and bacillary angiomatosis, has been proven to moderately, yet significantly upregulate the expression of *htrA* during infection of human microvascular epithelial cells (Resto-Ruiz *et al.*, 2000).

1.3.5.3 Cpx homologues in other pathogens

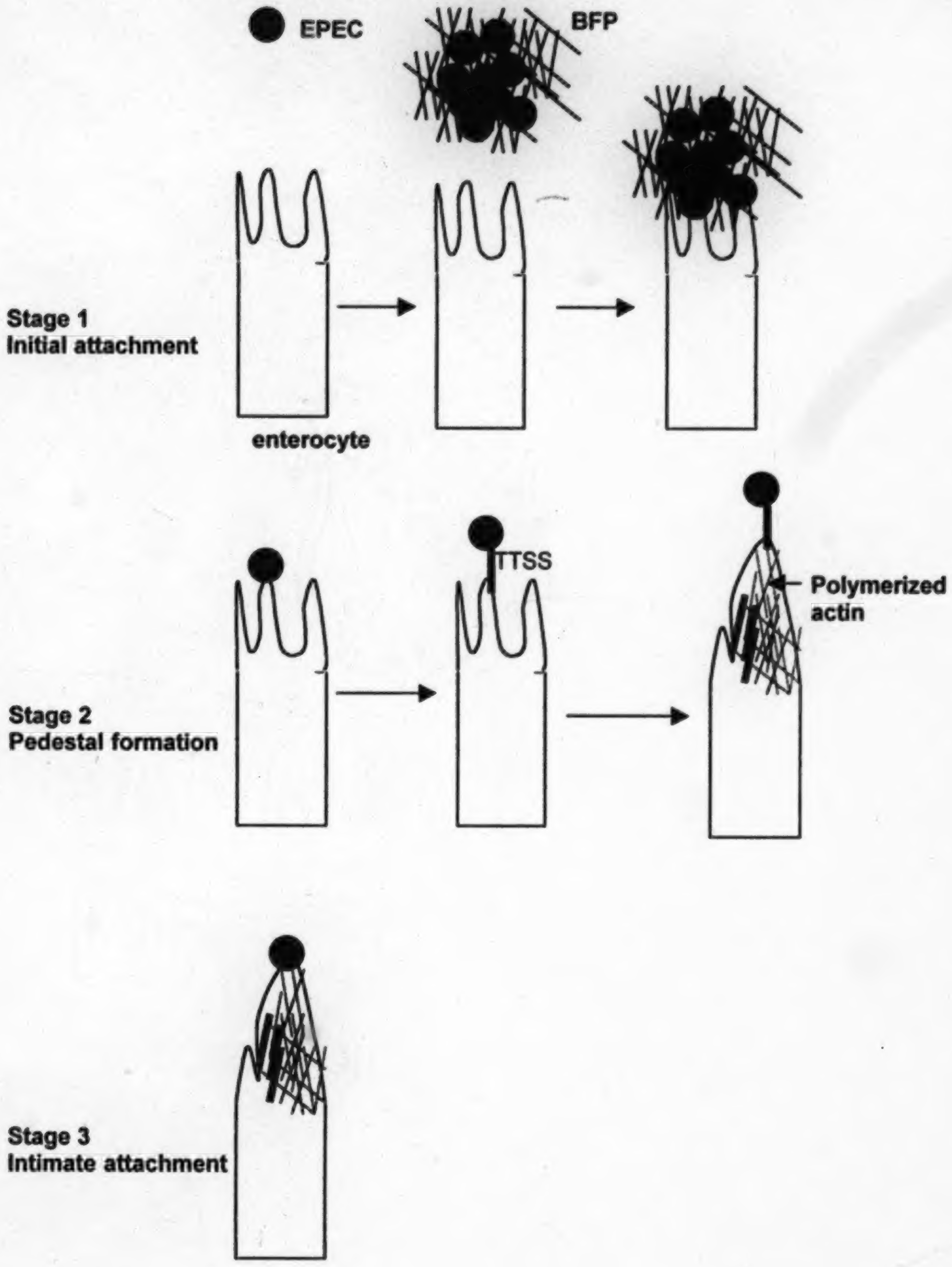
In addition to evidence that Cpx-regulated genes and their homologues are involved in virulence in some pathogenic organisms, there are also a number of proteins homologous to the Cpx signal transduction machinery involved in bacterial pathogenesis. In the bacterium *Shigella sonnei*, a close relative of *E. coli*, the Cpx system was proven to be involved in the pH dependent regulation of *virF*, a key positive transcriptional regulator of the *ipaBCD* genes (Nakayama and Watanabe, 1995; Nakayama and Watanabe, 1998). The products of these genes are introduced into epithelial cells through a type III secretory pathway and are essential for invasion (Watari *et al.*, 1995). De Wulf *et al.* (2000) analyzed the genomes of a large number of pathogenic bacteria using the *E. coli* protein and gene sequences for *cpxA*, *cpxR*, and *cpxP*, to search for homologues. They identified homologues to these three genes in *Y. pestis* and *S. typhi* (De Wulf *et al.*, 2000). Further characterization of the *cpx* homologues in *S. typhi* and *Y. pestis* demonstrated that CpxA, CpxR, and CpxP possess 100, 100, and 88% amino acid identity respectively, and 86, 87, 88% nucleotide identity respectively (De Wulf *et al.*, 2000). These data, combined with identical positioning of the CpxR-P recognition box in the *cpxRA* operon in *S. typhi*, suggest that the autogenous regulation characterized in *E. coli* also occurs in *S. typhi* (De Wulf *et al.*, 2000). Using the completed genome sequences of a number of pathogens, it was found that *Klebsiella pneumoniae* and *Shewanella putrefaciens* had genes homologous in both sequence and physical order to *cpxP, cpxR,* and *cpxA* (Raivio and Silhavy, 2001). As well, genes homologous to *cpxA* and *cpxR* were identified in *Vibrio cholerae, Actinobacillus actinomycetemcomitans*, and *Pasteurella multocida* (Raivio and Silhavy, 2001).

1.4 Enteropathogenic *Escherichia coli*

Enteropathogenic *E. coli* was the first pathogenic strain of *E. coli* discovered and is a major cause of infant diarrhea in the developing world (for a review see Donnenberg and Whittam, 2001). EPEC is similar to one of the two major groups of enterohemorrhagic *E. coli* (EHEC), a strain that can cause hemorrhagic colitis as well as severe renal and neurological symptoms (Frankel *et al.*, 1998). The difference in severity of the infections produced by EHEC and EPEC is mainly due to the secretion of the potent Shiga toxin by EHEC (Frankel *et al.*, 1998). A number of envelope-associated virulence determinants, such as type IV pili, adhesins and a type III secretory apparatus, are involved in EPEC pathogenesis (Donnenberg, 2000). Thus EPEC is often used as a model to study the pathogenicity of virulent *E. coli.* In addition to being very similar genetically to EHEC, it displays a similar infection process to a number of enteric pathogens such as EHEC, *Citrobacter rodentium* and *Hafnia alvei* (for a review see Celli *et al.*, 2000).

The classical model of EPEC infection is broken down into three major stages (Fig 1.4) (Donnenberg and Kaper, 1992). The first stage of infection is initial attachment to the host enterocyte. This initial attachment is mediated by type IV bundle-forming pili (BFP), structures of 4-7 nm in diameter that aggregate to form the rope-like clumps implied by its name (Giron *et al.*, 1991). BFP are responsible for EPEC binding to the host epithelial cells as microcolonies, a phenotype known as localized adherence (LA) (for a review see Nataro and Kaper, 1998). In the next step, the aggregation ceases, the bacteria disperse and induce changes in signal transduction in the host cell (for a review see Donnenberg and Kaper, 1992). These alterations to host signal transduction are mediated by the injection of bacterial effector molecules into the host cell by the bacterial envelope spanning type III secretory apparatus. The type III secreted effectors can cause alterations to enterocyte structure such as localized microvillus loss and actin rearrangements that lead to the formation of a pedestal-like structure underneath of the adhered bacterium. The pedestal is formed by first depolymerizing microvillus actin, then repolymerizing the actin under the bacterium. All of the genes involved in this process reside on a 35-kb pathogenicity island known as the locus of enterocyte effacement (LEE) and code for a type III secretory apparatus, several secreted effector proteins, and an outer membrane receptor called intimin (Elliott *et al.*, 1998). The final stage of infection is intimate adherence between EPEC and the surface of intestinal epithelial cells that causes the formation of a characteristic histopathology known as an attaching/effacing (A/E) lesion (for a review see Celli *et al.*, 2000; Vallance and Finlay, 2000). This intimate adherence is mediated by the LEE-encoded intimin and Tir proteins (Rosenshine *et al.*, 1996). Tir is translocated through the type III secretory system into the host cell where it becomes membrane localized and able to bind to intimin on the bacterial surface (Kenny *et al.*, 1997a).

Fig. 1.5. Model of EPEC pathogenesis. After entering the intestine, EPEC cells express bundle-forming pili (BFP), allowing the bacteria to bind together to form aggregates. These aggregates then bind to host enterocytes to complete the first stage of pathogenesis, localized adherence. The aggregates then disperse and the remaining bacteria cease to express BFP and construct a type III secretory apparatus (TTSS) which injects effector proteins into the host cell. These effectors result in a depolymerization of actin followed by a repolymerization of actin under the bacterium. The result of the second stage of pathogenesis is the formation of a large pedestal under the bacterium. The final stage of pathogenesis results in an intimate attachment between the bacterium and the enterocyte. This is mediated by the interaction of a bacterial outer membrane protein, intimin, and a secreted effector, Tir.

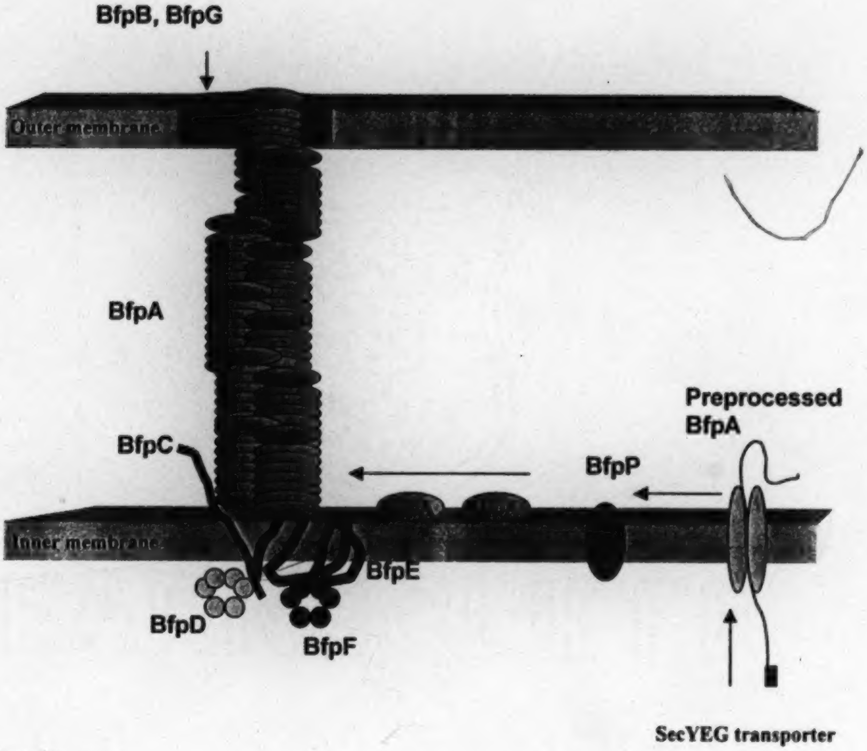

EPEC
BFP
Stage 1
Initial attachment
enterocyte
TTSS
Polymerized actin
Stage 2
Pedestal formation
Stage 3
Intimate attachment

1.4.1 Type IV Bundle-forming pili

The first stage in many bacterial infections is attachment to host cells followed by colonization. The microcolonies of EPEC observed on enterocyte surfaces are due to the reversible formation of type IV pili bundles (Donnenberg and Whittam, 2001). Type IV pili are thought to be assembled by a conserved pathway which resembles *sec*-dependent type II protein secretion and, in EPEC, requires the action of DsbA (Thanassi and Hultgren, 2000). In contrast to the chaperone-usher pathway, type IV pilus biogenesis is not well characterized. The 14 BFP coding genes are located on a large (60 MDa, 94 kbp) EPEC adherence factor (EAF) plasmid, and, when cured of the plasmid, EPEC cells lose their localized adherence pattern (Baldini *et al.*, 1983). As well, when the EAF plasmid is introduced into non-pathogenic *E. coli*, the strains gain the localized adherence phenotype (Baldini *et al.*, 1983).

BFP consist of repeats of a 19.5 kDa protein known as bundlin which is encoded by the *bfpA* gene in the *bfp* operon (Giron *et al.*, 1991). Bundlin is translated as a pre-peptide which is subsequently cleaved by the BfpP peptidase, a characteristic of type IV pilus biogenesis (see Figure 1.5) (Zhang *et al.*, 1994). However the cleaved leader sequence of BfpA is unusual in that it is hydrophilic (Zhang *et al.*, 1994). Interestingly, no adhesive moiety has been discovered to date for BFP and anti-BFP antibodies only partially block adherence suggesting that other factors are involved in adhesion (Elliott and Kaper, 1997). Recently the flagella and a large lymphotoxin, LifA, have also been implicated in the binding of EPEC to tissue culture cells (Giron *et al.*, 2002; Badea *et al.*, 2003).

Figure 1.6. A model of Bfp biogenesis. The unprocessed BfpA subunits are transported from the cytosol across the inner membrane by the SecYEG transporter. The BfpA subunits are processed by the BfpP prepilin peptidase and inner membrane localized BfpA subunits are then added onto the growing pilus by the inner membrane localized biogenesis machinery, BfpC, BfpD, BfpE, and BfpF. BfpD and BfpF are nucleotide-binding proteins that appear to play antagonistic roles in extension and retraction of the pilus. The growing pilus is then extruded through a pore in the outer membrane comprised of BfpB and BfpG.


BfpB, BfpG
Outer membrane
BfpA
Preprocessed
BfpA
BfpC
BfpP
Inner membrane
BfpE
BfpD
BfpF
SecYEG transporter

Another member of the operon, *bfpF*, codes for a cytoplasmic nucleotide binding protein that is required for an increase in bundle thickness followed by a decrease in adherence which leads to dispersal of EPEC from microcolonies (Knutton *et al.*, 1999; Anantha *et al.*, 1999). BfpF has homology to PilT, a putative nucleotide binding protein which provides energy for the retraction of the *Pseudomonas aeruginosa* type IV pilus through ATP hydrolysis (Anantha *et al.*, 1998). The second ORF in the operon encodes the BfpB protein, an outer membrane lipoprotein that functions in BFP biogenesis after BfpA translation and processing (Ramer *et al.*, 1996). The protein shows homology to PulD, an outer membrane protein of the general secretory pathway that may form a channel, allowing protein translocation (Hardie *et al.*, 1996). BfpE is a cytoplasmic membrane spanning protein of poorly understood function required for pilus biogenesis (Blank and Donnenberg, 2001). The products of the eight aforementioned genes are the only well characterized members of the 14 *bfp* genes on the EAF plasmid. The gene *bfpD* is believed to encode a putative nucleotide binding factor that plays an antagonistic role to the BfpF protein in providing energy for pilus elongation (Stone *et al.*, 1996; Crowther *et al.*, 2004). Western analysis of EPEC cellular compartments revealed that BfpG is an outer membrane protein, BfpU and BfpH are localized to the periplasm, and BfpI, BfpJ, BfpK, and BfpL are inner membrane localized (Schmidt *et al.*, 2001; Ramer *et al.*, 2002; Schreiber *et al.*, 2002).

To determine the cellular location of BfpC a series of 3' deletions of the *bfpC* gene were fused to an alkaline phosphatase/β-galactosidase reporter (Crowther *et al.*, 2004). Alkaline phosphatase requires a disulfide bond and is only active in the periplasm while β-galactosidase requires the omega-fragment for complementation and is only active in the cytoplasm (Crowther *et al.*, 2004). BfpC was determined to be a bitopic membrane protein with the first 164 residues in the cytoplasm and residues 188-402 localized in the periplasm (Crowther *et al.*, 2004). Yeast two hybrid analysis of BfpC, BfpD, BfpE and BfpF revealed that the cytoplasmic domain of BfpC interacted with BfpD and the N-terminus of BfpE (Crother *et al.*, 2004). As BfpD, E, and F all interact with themselves in the assay, they appear to be multimeric in the cell (Crowther *et al.*, 2004). BfpD was also demonstrated to interact with the N-terminus of BfpE while BfpF interacts with a second cytoplasmic domain of BfpE (Crowther *et al.*, 2004).

BfpC, BfpD, and BfpE form a stable complex together and when one of these proteins is missing the stability of the remaining proteins is altered (Crowther *et al.*, 2004). BfpC and BfpE appear to recruit BfpD to the cell membrane since, in the absence of these proteins, BfpD is only found in the soluble fraction of a cell lysate (Crowther *et al.*, 2004). Overexpressing the N-termini of either BfpD or BfpE inhibits BFP biogenesis, presumably by competing with wild-type BfpD or BfpE for interaction with essential components of the BFP assembly apparatus (Crowther *et al.*, 2004). This overexpression does not affect retraction, suggesting that these proteins interact with other proteins involved in biogenesis but, as demonstrated in the yeast two-hybrid assay, do not interact with BfpF (Crowther *et al.*, 2004). Overexpressing the cytoplasmic loop of BfpE inhibited BFP retraction, likely by sequestering BfpF and preventing it from interacting with full-length BfpE (Crowther *et al.*, 2004). To determine the identities of proteins involved in the BFP complex *in vivo*, Hwang *et al.* (2003) used a strain carrying a BfpB-His_6 fusion protein and chemically crosslinked the BFP complex *in situ*. The BFP complex was then purified using both nickel and BfpB-antibody affinity columns and immunoblotting using Bfp specific antisera was performed (Hwang *et al.*, 2003). Of the fourteen Bfp proteins, ten were shown to interact *in vivo*: BfpA, BfpB, BfpC, BfpD, BfpE, BfpF, BfpG, BfpJ, BfpL, and BfpU (Hwang *et al.*, 2003). As these proteins have been localized to the inner membrane, periplasm, and outer membrane, the BFP complex appears to be a membrane spanning structure (Hwang *et al.*, 2003). Using both BfpF and BfpL antisera, immunofluorescence localized the BFP to the poles of the cell, similar to the type IV pili of *Pseudomonas aeruginosa* (Hwang *et al.*, 2003).

1.4.2 Type I fimbriae

EPEC strain E2348/69, the model pathogenic strain of this organism, has been shown to produce only two types of fimbrial adhesins: BFP and type I fimbriae (Elliott and Kaper, 1997). When volunteers were infected with E2348/69, an immune response against type I fimbriae was noted and high titres of anti-fimbrial antibodies were correlated with a milder form of infection (Karch *et al.* 1987). This protective effect suggested that type I pili may play a role in EPEC pathogenicity. However, further studies found that the presence or absence of type I fimbriae had no effect on EPEC localized adherence. Thus the role of type I fimbriae in pathogenicity is unknown (Elliott and Kaper, 1997).

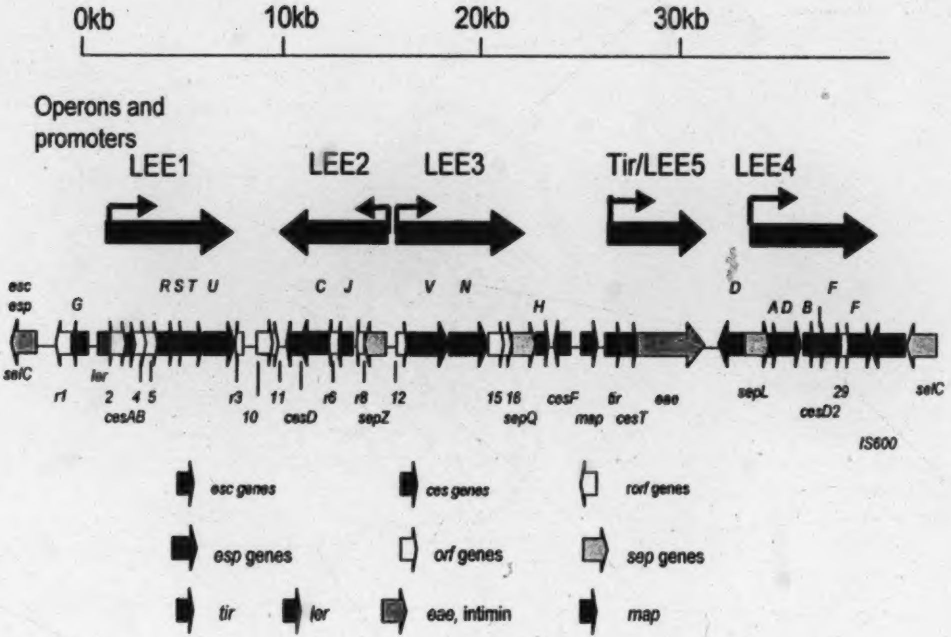
1.4.3 Locus of enterocyte effacement

While the genes required for initial attachment are located on the EAF plasmid, the genes required for the formation of the A/E lesion characteristic of EPEC reside on a 35 kb pathogenicity island (PAI) known as the locus of enterocyte effacement or LEE

(McDaniel and Kaper, 1997). The LEE has been identified as a pathogenicity island because: i) it encodes virulence-associated genes, ii) it is absent in avirulent *E. coli* strains, iii) it shows conserved insertion in the E2348/69 genome at minute 82 of the K-12 chromosome, near the selenocysteine tRNA locus (*selC*), and iv) it has a G+C content of 38% compared to the 50.8% G+C content of the *E. coli* chromosome, which indicates it originated in another species (Frankel *et al.*, 1998). Interestingly, the PAI of UPEC is also inserted at minute 82 of the K-12 chromosome, adjacent to the same locus (Frankel *et al.*, 1998).

The E2348/69 LEE carries 41 genes arranged in 5 polycistronic operons, (as shown in Figure 1.6) which were identified by primer extension analysis and a combination of transcriptional fusions and RT-PCR analysis (Elliott *et al.*, 1998; Mellies *et al.*, 1999; Sanchez-SanMartin *et al.*, 2001). The first operon, *LEE1*, contains both the *escRSTU* genes, which are involved in type III secretion, and the H-NS like *ler* gene which encodes a regulatory protein (Elliott *et al.*, 1998; Mellies *et al.*, 1999). The second operon, *LEE2*, contains the *escCJ* and *sepZ* genes, also involved in type III secretion, along with the *cesD* gene which is a chaperone molecule for the secreted EspD and EspB proteins, which are responsible for forming pores in the enterocyte membrane (Elliott *et al.*, 1998). The third operon, *LEE3*, contains *escVN*, also involved in type III secretion, while the fourth operon, *LEE4*, contains the *espADBF* genes, which code for proteins secreted through the type III apparatus (Elliott *et al.*, 1998). Also in the E2348/69 LEE, in a fifth operon, *LEE5*, are the *tir* and *eae* genes, which interact to mediate intimate attachment of the pathogen to the enterocyte and the *cesT* gene which codes for a Tir chaperone (see below) (Sanchez-San Martin *et al.*, 2001).

Figure 1.7. The enteropathogenic *Escherichia coli* locus of enterocyte effacement. Adapted from Zhu *et al.*, 2001. The LEE is a 35 kb pathogenicity island comprised of 5 polycistronic operons, transcribed in the directions indicated by the arrows. The operons contain the *esc* and *sep* genes, which code for the type III secretory apparatus, as well as the *esp* genes, which encode translocated proteins. The *ces* genes encode chaperones, the *ler* gene product is a positive regulator of *LEE2, LEE3, LEE4* and *LEE5,* while the *tir* gene product is the secreted receptor for the *eae* gene product. The EPEC LEE is inserted in the *selC* locus of the EPEC chromosome.

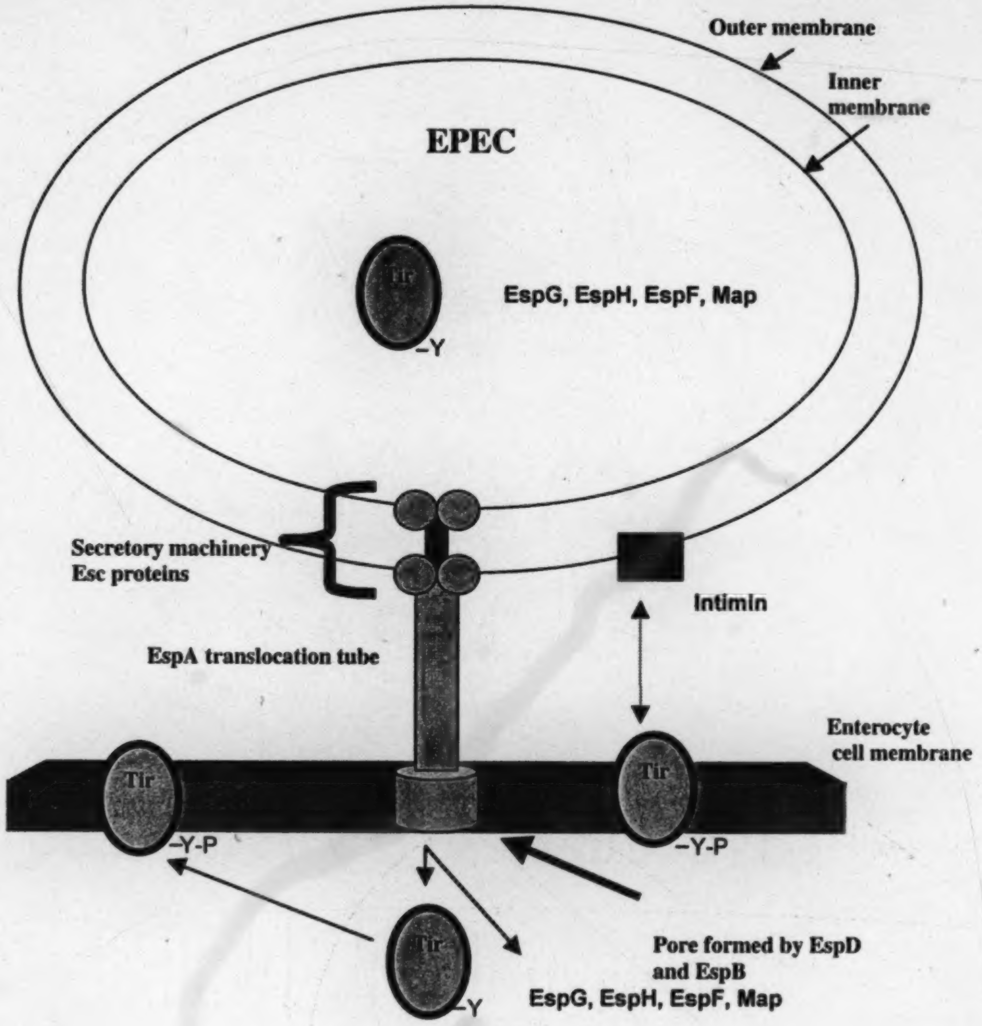

0kb
10kb
20kb
30kb
Operons and promoters
LEE1
LEE2
LEE3
Tir/LEE5
LEE4
esc
esp
G
R S T U
C J
V N
H
D
F
A D B
F
selC
ler
r1
2
4
5
cesAB
r3
10
11
cesD
r6
r8
sepZ
12
15 16
sepQ
cesF
map
tir
cesT
eae
sepL
29
cesD2
selC
IS600
esc genes
ces genes
rorf genes
esp genes
orf genes
sep genes
tir
ler
eae, intimin
map

1.4.4 Type III secretion

Gram negative bacterial pathogens are able to transport proteins across their inner and outer membranes and through the host cell cytoplasmic membrane using a type III secretory apparatus, thus allowing delivery of effector molecules into targeted cells (see Figure 1.7) (Donnenberg and Whittam, 2001; Smith and Hultgren, 2001). The EPEC proteins involved in type III secretion fall into two classes; secreted effectors and the actual components of the secretory apparatus (Donnenberg and Whittam, 2001). EspA forms the needle-like tranlocation tube while EspB and EspD work in concert to form pores in the cytoplasmic membrane of the target cell (Ide *et al.*, 2001), as shown in Figure 1.7. The *esc* and *sep* genes code for the machinery of the apparatus responsible for transporting the EspA, EspB and EspD proteins from the cytoplasm to the external environment, presumably through the generation of a pore to facilitate translocation (Sekiya *et al.*, 2001). Cytosolic chaperones are required for the effective translocation of secreted proteins. The CesD protein is required for the secretion of EspB and EspD, while CesT is necessary for the secretion of Tir as well as Map, making it the first bivalent chaperone in its class (Wainwright and Kaper, 1998; Elliott *et al.*, 1999; Abe *et al.*, 1999; Creasey *et al.*, 2003a). CesAB, formerly known as Orf3, is another chaperone required for stability of EspA in the bacterial cytoplasm and proper formation of EspA filaments (Creasey *et al.*, 2003b). CesAB also mediates translocation of EspB and a strain carrying a mutation in *cesAB* fails to cause A/E lesions and cannot bind to or cause lysis of erythrocytes (Creasey *et al.*, 2003b).

The final stage of EPEC infection is intimate adherence of the bacterium to the host cell which is mediated through the interaction of a type III secreted protein and its

Figure 1.8. Type III secretion in EPEC. Adapted from Vallance and Finlay (2000). The products of the LEE-encoded *esc* genes form the type III secretory apparatus that spans the inner membrane, the periplasmic space, and the outer membrane. The EspA protein forms a needle-like translocation tube which facilitates transportation of the bacterial effectors through the extracellular milieu, and the pore formed in the enterocyte membrane by the EspD and EspB proteins. Effectors include the Tir protein, which is phosphorylated (P) at a conserved tyrosine residue (Y) and is subsequently membrane localized, where it acts as a receptor for the bacterial associated intimin protein. Intimin association with Tir mediates the intimate adherence characteristic of the third stage of infection. Other secreted effectors include Map, which is involved in filipodia formation as well as inducing dysfunction of mitochondria, EspF, which triggers apoptosis, EspH, a cytoskeletal modifier, and EspG which plays an accessory, undefined role in pathogenesis.


Outer membrane
Inner membrane
EPEC
Tir
–Y
EspG, EspH, EspF, Map
Secretory machinery
Esc proteins
Intimin
EspA translocation tube
Enterocyte cell membrane
Tir
–Y-P
Tir
–Y-P
Tir
–Y
Pore formed by EspD and EspB
EspG, EspH, EspF, Map

cognate receptor. The product of the *eae* gene, intimin, is a 94 kDa outer membrane protein that binds to a 90 kDa receptor protein in the host cell membrane (Rosenshine *et al.*, 1996). Originally believed to be a host-derived receptor, it was subsequently found to be the bacterial Tir protein. This is the first known case of a pathogen injecting its own receptor through a type III apparatus into a mammalian cell (Kenny *et al.*, 1997; Vallance and Finlay, 2000). The bacterial form of Tir is a 78 kDa protein, which is known to be phosphorylated on a tyrosine residue upon entry into enterocytes (Kenny *et al.*, 1997a; Vallance and Finlay, 2000). In addition to mediating tighter adherence, the phosphorylated Tir protein is required for the cytoskeletal rearrangements responsible for pedestal formation and loss of microvilli (Kenny, 1999).

Other translocated proteins include Map (mitochondrial associated protein), EspF, EspG, and EspH. Once delivered to the host cell, Map is targeted to the portion of the mitochondrial membrane underlying the area where the infecting bacterium has bound to the cellular membrane and causes loss of mitochondrial membrane potential (Kenny and Jepson, 2000). The effect of Map on cytoskeletal function is independent of its mitochondrial disruption ability. Thin, actin-rich cytoplasmic extensions termed filopodia are still produced in HeLa cells when infected with an EPEC strain which carries a plasmid encoding a Map protein with a Tir N-terminal sequence (Kenny *et al.*, 2002). This protein is secreted by EPEC, but not targeted to mitochondria (Kenny *et al.*, 2002). The upregulation of filipodia formation is mediated in a yet undefined manner through the host protein Cdc42, a regulatory GTPase protein (Kenny *et al.*, 2002). Interestingly, in addition to inducing actin rearrangements, Tir posseses an arginine finger (GXLR) motif which downregulates filipodia formation, putatively through the inactivation of a GTPase (Kenny *et al.*, 2002). As with the instigation of pedestal formation, inhibition of filipodia is dependent on the presence of Intimin-Tir interaction (Kenny *et al.*, 2002). After delivery into the host cell the EspH effector localizes under the bacterium and modulates the host cell cytoskeleton (Tu *et al.*, 2003). Infection of HeLa cells with an EPEC strain carrying an insertionally inactivated *espH* gene resulted in increased filipodia formation while pedestal formation was hindered (Tu *et al.*, 2003). EPEC also translocates the proline rich EspF protein which is believed to interact with host signaling molecules to induce apoptosis in intestinal enterocytes (Crane *et al.*, 2001). EspG is structurally and functionally homologous to *Shigella* VirA, which plays an undefined accessory role in invasion (Elliott *et al.*, 2001). A lambdoid phage-encoded secreted effector, Cif, has recently shown to be secreted by the EPEC type III secretory system (Marches *et al.*, 2003.) When introduced into HeLa cells Cif causes formation of stress fibres and results in a G_2/M cell cycle arrest (Marches *et al.*, 2003).

As EPEC is a strict human pathogen there is a paucity of appropriate models to study *in vivo* functions of virulence determinants. The LEE is conserved in other enteric pathogens such as EHEC and *Citrobacter rodentium*. A recent study by Deng *et al.* (2004) analyzed the effects of non-polar mutations in each of the 41 genes in the *C. rodentium* LEE *in vivo* using a murine model, the natural host for this organism. Interestingly the entire LEE was required for full virulence, though the effect of the deletions on *in vivo* virulence ranged from abolition of virulence to moderate attenuation (Deng *et al.*, 2004). This was in contrast to the pathogenicity islands of *Salmonella*, which contain a great deal of redundancy and thus can withstand mutation without a change in virulence (for a summary of results see Table 1.1) (Deng *et al.*, 2004).

Analysis of EspB and Tir secretion by all 41 mutants revealed that, as expected, deletions of the *espB* and *tir* genes as well as the Tir chaperone *cesT* abrogated secretion (Deng *et al.*, 2004). Unexpectedly, deletion of *orf11* abolished secretion as well (Deng *et al.*, 2004). This predicted polypeptide has 23% homology to CaiF, an *Enterobacteriaceae* transcriptional activator, and is a positive regulator. The *orf11* gene product, renamed GrlA, acts upstream of Ler, as demonstrated by examining secretion of Tir and EspB in in a Δ*ler* Δ*grlA* double mutant with either Ler or GrlA being supplied in *trans* (Deng *et al.*, 2004). As secretion was restored only by expressing Ler, GrlA appeared to act before Ler in the regulatory cascade (Deng *et al.*, 2004). The product of *orf10,* now named GrlR, functions as a negative regulator as a Δ*grlR* mutant has increased levels of *ler* transcription and overexpression of GrlR results in decreased Tir and EspB secretion (Deng *et al.*, 2004).

Although sequence similarity had identified the *esc* genes as likely components of the type III secretory apparatus, with the exception of *escF, escC, escD, escN* and *escV,* a functional role in secretion had not been proven until this study (Deng *et al.*, 2004). Translocators are proteins involved in delivering effectors to the host cell but are also secreted via the TTSS themselves. The 10 *esc* genes, as well as *orf2*, *orf4*, *orf5*, *rorf3*, *rorf8*, *orf12*, *orf15*, *sepQ,* and *orf29*, which affected secretion of both translocators and effectors and *orf3*, *rorf6*, *orf16*, and *sepL,* which predominantly affected translocator secretion, were required for type III secretion (Deng *et al.*, 2004). The genes of unknown function in the LEE transcribed in a rightward direction are designated *orfs*, while the unknown genes transcribed in the opposite direction are referred to as *rorfs*. Mutations in

Table 1.1. Functional analysis through deletion of the 41 LEE encoded genes in *C. rodentium*

Deleted Gene	Operon location	Function[a]	Type III Secretion	Effect on virulence[b]
WT	-	-	+	+++
rorf1	-	Unknown	+	+
rorf2 (espG)	-	secreted effector	+/no EspG	+
ler	LEE1	Positive virulence regulator	-	-
orf2	LEE1	TTSS	-	-
orf3 (cesAB)	LEE1	EspA, EspB chaperone	attenuated-EspB, EspD	-
orf4	LEE1	TTSS	-	-
orf5	LEE1	TTSS	-	-
escR	LEE1	TTSS	-	-
escS	LEE1	TTSS	-	-
escT	LEE1	TTSS	-	-
escU	LEE1	TTSS	-	-
rorf3		Putative TTSS assembly	attenuated-all effectors	+/-
orf10 (grlR)		Negative virulence regulator	+	++
orf11(grlA)		Positive virulence regulator	-	-
cesD	LEE2	EspD chaperone	No EspD	-
escC	LEE2	TTSS	-	-
rorf6	LEE2	Control-translocator secretion	No translocator secretion	-
escJ	LEE2	TTSS	-	-
rorf8	LEE2	TTSS	-	-
sepZ	LEE2	Unknown	+	+/-
orf12	LEE3	TTSS	-	-
escV	LEE3	TTSS	-	-
escN	LEE3	TTSS	-	-
orf15	LEE3	TTSS	-	-
orf16	LEE3	Translocator secretion	attenuated-translocators	+/-
sepQ	LEE3	TTSS	-	-
orf18(espH)	LEE3	secreted effector	+/no EspH	++
rorf10(cesF)		EspF chaperone	attenuated-EspF	+
orf19(map)		secreted effector	+/no Map	++
tir	LEE5	secreted effector	+/no Tir	-
cesT	LEE5	Tir chaperone	attenuated-Tir	-
eae	LEE5	Adhesion to Tir	+	-
escD		TTSS	-	-
sepL	LEE4	Control of translocator secretion	No translocator secretion	-
espA	LEE4	Translocation tube	+/no Espa	-
espD	LEE4	Pore in target cell membrane	+/no EspD	-
espB	LEE4	Pore in target cell membrane	+/noEspB	-
orf27(cesD2)	LEE4	EspD chaperone	attenuated-EspD	+/-
escF	LEE4	TTSS	-	-
orf29	LEE4	TTSS	-	-
espF	LEE4	secreted effector	attenuated-EspF	+

a- wildtype(+), absence (-), or attenuation (+/-) of type III secretion. Attenuation/absence of specific *secreted proteins is indicated after the "/"*

b-Wildtype virulence (+++), slight attenuation in disease or alterations in virulence/colonization (++), moderate attenuation in colonization and colonic hyperplasia (+), severe attenuation of colonization, no colonic hyperplasia (+/-), or avirulence/no disease (-)

these genes appeared to have differential effects on secretion. Deletion mutants of *orf16* and *orf3* both secreted Tir normally but the *orf16* mutant secreted wildtype levels of EspD but reduced levels of EspB and EspA while the *orf3* mutation reduced levels of all three translocators (Deng *et al.*, 2004). Deletions of *sepL* and *rorf6* increased secretion of Tir and NleA, a recently identified non-LEE encoded effector (Deng *et al.*, 2004). This secretion increase was dependent on the presence of a functional secretory apparatus and it appears that SepL and Rorf6 may function in an additional level of regulation in secretion.

Next, the effect of each LEE mutation on pedestal formation was examined. The positive regulators *ler* and *orf11*, the type III secretory genes, the translocator genes, EspA, EspB, and EspD, as well as *tir, eae,* and *cesT* were all required for proper pedestal formation while *orf16* and *cesD,* which encode proteins involved in the secretion of translocators had only sporadic pedestal formation (Deng *et al.*, 2004). Finally, each deletion mutant was used in a mouse infection model to analyze the function of each gene in virulence. As with pedestal formation, Ler and GrlA, as well as all of the type III secretory genes were essential for virulence, while *grlR* deletions showed a small but significant decrease in both colonization and the level of colonic hyperplasia they caused (Deng *et al.*, 2004). Colonic hyperplasia is characterized by a 3-4 fold increase in the number of crypt cells in the lower colon, which interestingly, is frequently observed in paediatric EPEC cases, as well as soft stool and increased fluid in the colon (for a review read Luperchio and Schauer, 2001; Nataro and Kaper, 1998). Tir and Eae were the only effectors essential for full virulence, while *espF* and *espG* deletions had moderate decreases in colonization and colonic hyperplasia (Deng *et al.*, 2004). The effects of *map* and *espH* deletions were very minor and mutations in chaperones had the same effect as mutations in the associated effector (Deng *et al.*, 2004). Mutations in *rorf3, orf16, cesD2,* and *sepZ* abolished colonic hyperplasia but did not affect colonization. Only a minor attenuation of virulence was observed in *grlR, map, cesF, rorf1, espG, espF,* and *espH* deletions (Deng *et al.*, 2004).

To isolate novel effectors secreted by the *Citrobacter* LEE, a GrlA overexpression plasmid was used, which increased secreted EspA, EspB, and EspD 300 fold (Deng *et al.*, 2004). Through proteomic analysis of secreted proteins seven novel secreted proteins were identified: NleA, NleB, NleC, NleD, NleE, NleF, and NleG, all of which, with the exception of NleG, are conserved in EHEC (Deng *et al.*, 2004). Analysis of the partially sequenced EPEC genome has identified homologues of the EHEC NleA-F genes with 89-95% sequence similarity (Deng *et al.*, 2004).

1.4.5 Regulation of virulence factors

The plasmid-encoded *bfp* genes as well as the Ler-regulated genes on the pathogenicity island are said to comprise the Per regulon. The products of the *bfpTWV* genes (also known as *perABC)* comprise plasmid-encoded regulators (BfpTWV/PerABC) required for the expression of both BFP and Ler (Mellies *et al.*, 1999; Tobe *et al.*, 1996). PerA is a member of the AraC family of transcriptional activators, while PerB and PerC enhance PerA activity through a currently uncharacterized mechanism (Gomez-Duarte and Kaper, 1995; Tobe *et al.*, 1996). The Per-regulated expression of *bfp* genes on the plasmid has been shown to be driven by a promoter upstream of *bfpA* that is activated at elevated temperatures and diminished ammonium levels (Tobe *et al.*, 1996; Puente *et al.*, 1996). A screen searching for regulators of *per* expression isolated the GadX protein, a putative member of the AraC/XylR family of transcriptional regulators (Shin *et al.*, 2001). The regulator is located downstream of *gadA*, a gene that codes for a glutamate decarboxylase enzyme involved in the acid response (Shin *et al.*, 2001). The GadX protein was shown to bind to the *perA* and *gadA* promoters (Shin *et al.*, 2001). Under conditions where virulence genes are normally expressed, GadX was shown to decrease *perA* expression (Shin *et al.*, 2001). As the GadX repressor is active in acidic conditions, it may repress virulence determinant expression in the acidic environment of the stomach and ensure proper expression occurs in the less acidic environment of the small intestine (Shin *et al.*, 2001). The EPEC quorum sensing system also indirectly activates a *perA:lacZ* reporter in an E2348/69 background, but not in an MC4100 background, suggesting that the activation is relayed through an EPEC specific regulator (Sperandio *et al.*, 1999).

PerA is required to express intimin, the *LEE1* operon and *bfpA* (Gomez-Duarte and Kaper, 1995; Mellies *et al.*, 1999). Per-dependent activation of the *LEE1* operon activates expression of the *ler* gene (LEE encoded regulator), the product of which subsequently increases transcription from *LEE2, LEE3, LEE4,* and *LEE5* (Mellies *et al.*, 1999; Haack *et al.*, 2003). The nucleoid associated regulatory protein Fis was shown to be required for transcription of both *LEE4* and the *ler* gene (Goldberg *et al.*, 2001). In addition to indirectly regulating *perA* expression the quorum sensing system has a direct positive effect on *LEE1* and *LEE2* transcription as pre-conditioned media can enhance activity of *LEE1* and *LEE2 lacZ* reporters in MC4100 (Sperandio *et al.*, 1999). IHF, another nucleoid associated regulatory protein, was shown to directly activate *LEE1* and *espG* transcription through flow cytometry of *gfp* fusions in E2348/69 and E2348/69 *ihfA:kn* backgrounds (Friedberg *et al.*, 1999). IHF also mediates repression of the flagellar activator *flhDC* and E2348/69 *ihfA:kn* mutants are hypermotile (Yona-Nadler *et al.*, 2003). A novel ribosome binding GTPase, BipA, appears to activate *ler* transcription indirectly, as a *bipA* mutation results in decreased *ler* transcript levels, yet purified BipA does not bind to a *ler* promoter fragment in gel mobility shift assays (Farris et al., 1998; Grant et al., 2003). As BipA has been demonstrated to bind to ribosomes and possesses enhanced GTPase activity after binding, it has been postulated that it functions as a translational regulator (Grant *et al.*, 2003). It is possible that BipA enhances the translation of a regulatory protein, which in turn enhances the expression of *ler*.

Ler is believed to bind to the promoter of *LEE2*, changing DNA topology and allowing transcription of the divergent operons *LEE2* and *LEE3* (Sperandio *et al.*, 2000). Furthermore, the Ler binding sites overlap with the binding site of the inhibitory nucleoid associated protein H-NS. Ler binding is believed to displace H-NS, alleviating its bidirectional silencing effect on the *LEE2* and *LEE3* promoters, as well as on the *LEE5* promoter (Bustamente *et al.*, 2001; Haack *et* al., 2003). A series of *tir-CAT* reporter fusions demonstrated that repression of *tir* transcription in rich media and at low temperature is independent of H-NS repression and involves sequences within the promoter region (Sanchez-SanMartin *et al.*, 2001). Repression of *tir* transcription due to the presence of ammonium in the medium is dependent on a sequence upstream of the promoter that overlaps with the H-NS interaction site, suggesting that this repression is H-NS mediated (Sanchez-SanMartin *et al.*, 2001).

It is believed that initial contact with epithelial cells induces *de novo* protein synthesis in EPEC, which then activates type III secretory genes, as protein synthesis is required for the A/E phenotype (Rosenshine *et al.*, 1996). The formation of A/E lesions is growth phase dependent as the ability to form A/E lesions was only detected in mid-logarithmic phase cells (Rosenshine *et al.*, 1996). In agreement with this observation, intimin levels increase during exponential phase, but are low in stationary phase, after the bacterium adheres to the epithelial cell (Knutton *et al.*, 1997; Rosenshine *et al.*, 1996). Initiation of A/E lesion formation is also temperature dependent as cells grown at 37°C caused characteristic A/E lesions on HeLa cells whereas EPEC grown at 28°C did not (Rosenshine *et al.*, 1996). Interestingly, EPEC grown at 37°C, but added to HeLa cells at 28°C, still induced A/E lesion formation suggesting that initiation of A/E lesion formation (but not the process itself) is temperature dependent (Rosenshine *et al.*, 1996). Control of EPEC virulence is very complicated and involves numerous LEE and non-LEE encoded regulators working at many levels.

1.5 Proposed Research Project

The goal of this project was to discover if a link exists between the Cpx two-component signal transduction system and EPEC pathogenicity. As outlined in this chapter, the Cpx system senses periplasmic stress and upregulates folding and degrading factors. It is hypothesized that when P pilus binding to epithelial cells blocks pilus elongation, misfolded subunits accumulate in the periplasm (Mulvey *et al.*, 1998; Raivio, 2001). It is known that misfolded P pilus subunits in the periplasm activate the Cpx pathway, leading to upregulation of folding and degrading factors (Hung *et al.*, 2000).

The fact that putative CpxR binding sites have been located upstream of the UPEC pathogenicity island leads to the notion that the Cpx pathway may sense attachment to a host cell and then upregulate the expression of virulence factors. Type IV pili such as BFP undergo twitching motility, a process that involves extension and retraction of pili (Bieber *et al.*, 1998; Knutton *et al.*, 1998). Upon attachment to intestinal enterocytes this process may also lead to the accumulation of misfolded type IV pilus subunits in the inner membrane, which the Cpx system is hypothesized to sense. Further, a variety of Cpx-regulated DsbA and DegP homologues are implicated in virulence in a number of pathogens and the *cpx*RA genes have been shown to be involved in regulation of pathogenesis in *Salmonella* and *Shigella*. Essentially the goal of this study was to determine whether the Cpx system acts to control virulence genes or regulate factors necessary for the expression and assembly of envelope-associated virulence factors.

The creation of E2348/69 strains carrying *nlpE* and *cpxR* overexpression vectors combined with the availability of anti-Tir and anti-EspB antibodies allowed the use of techniques such as immunoblot and ELISA analysis to examine the effect of Cpx activation on secretion. This, combined with the use of a hemolysis assay demonstrated that Cpx activation resulted in an increase in functional virulence determinant secretion. The fact that the LEE encoded genes involved in EPEC pathogenicity after initial attachment are found in operons made it extremely conducive to study expression. The transcription of one target from each operon was analyzed in the presence and absence of Cpx activation to determine whether the Cpx system had any transcriptional effect on LEE gene expression and these results were verified through the use of luciferase reporter fusions.

2. MATERIALS AND METHODS

2.1 Bacterial strains, plasmids, and growth conditions

2.1.1 *E. coli* strains

All *E. coli* strains used in this study are listed in Table 2.1.

2.1.2 Cloning vectors used in this study

All cloning vectors used in this study are listed in Table 2.2

2.1.3 Recombinant plasmids constructed and used

All recombinant plasmids, cloning strategies and their uses are listed in Table 2.3. To create pJW15, pNLP10 was digested with *Pac*I to remove the pSC101 origin. A *PacI* digested PCR fragment containing the p15a origin, derived from pACYC184, was then ligated into the *PacI* digested pNLP10 vector.

2.1.4 Growth and maintenance of *E. coli* strains

Liquid *E. coli* cultures were grown in LB medium at 37°C on an orbital shaker at 225 rpm. Solid cultures were grown on LB medium (5g/L yeast extract (Gibco), 10g/L tryptone (Gibco), 10g/L NaCl (EM Science) with 1.5% agar (w/v)) at 37°C. When required, ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), tetracycline (25 μg/ml), or spectinomycin (20 μg/ml) were used. *E. coli* frozen stocks

Table 2.1. ***Escherichia coli* strains**

E. coli strain	Genotype	Reference or Source
ALN88	E2348/69 *cpxR:kn*	A. Nevesinjac
CFM 14-2-1	E2348/69 *sepB*:Tn*phoA*	Donnenberg *et al.* (1990)
E2348/69	Prototype O127:H6 EPEC strain	Levine *et al.* (1978)
E2348/69Δler	E2348/69 carrying an in frame deletion of *ler*	Bustamente *et al.* (2001)
JPN15	E2348/69 cured of the EAF plasmid	Jerse *et al.* (1990)
TOP 10	F- *mcrA* Δ(*mrr-hsdRMSmcrBC*) ϕ80*lacZ*DM15 Δ*lacX*74 *endA1 recA1 deoR* Δ(*ara-leu*)*7697 araD139 galU galK nupG rpsL*)	Invitrogen
TR132	MC4100 *cpxR:spc* TnCam conferring Ara^{R}	T. Raivio
JLM 164	MC4100 Φ*LEE1::lacZ*	V. Sperandio; Mellies *et al.* (1999)
JLM 166	MC4100 Φ*LEE2::lacZ*	V. Sperandio; Mellies *et al.* (1999)
JLM 172	MC4100 Φ*LEE3::lacZ*	V. Sperandio; Mellies *et al.* (1999)
JLM 165	MC4100 Φ*LEE4::lacZ*	V. Sperandio; Mellies *et al.* (1999)
JW1	E2348/69(pBAD18)	This study
JW2	E2348/69(pND18)	This study
JW3	E2348/69(pROX)	This study
JW4	ALN88(pROX)	This study
JW11	ALN88(pBAD18)	This study
JW12	JPN15(pBAD18)	This study
JW13	JPN15(pND18)	This study
JW20	TR132(pROX)	This study
JW21	ALN88(pND18)	This study
JW29	E2348/69Δler (pBAD18)	This study
JW 58	JW20 Φ*LEE1::lacZ*	This study
JW 59	JW20 Φ*LEE2::lacZ*	This study
JW 60	JW20 Φ*LEE3::lacZ*	This study
JW 61	JW20 Φ*LEE4::lacZ*	This study
JW62	E2348/69Δler (pND18)	This study
JW63	E2348/69Δler (pROX)	This study
JW68	JW20(pJW2)	This study
JW81	JW20(pJW5)	This study
JW84	JW20(pJW6)	This study
JW89	JW20(pJW8)	This study
JW116	JW20(pJW13)	This study
JW117	JW20(pJW12)	This study
JW128	JW1(pJW15)	This study
JW129	JW2(pJW15)	This study
JW130	JW3(pJW15)	This study
JW134	JW20(pNLP10)	This study

E. coli strain	Genotype	Reference or Source
JW135	JW20(pJW3)	This study
JW136	JW20(pJW15)	This study
JW137	JW20(pJW17)	This study
JW154	JW1(pJW19)	This study
JW155	JW2(pJW19	This study
JW156	JW3(pJW19)	This study
JW157	JW1(pJW20)	This study
JW158	JW2(pJW20)	This study
JW159	JW3(pJW20)	This study
JW160	JW1(pJW22)	This study
JW161	JW2(pJW22)	This study
JW162	JW3(pJW22)	This study
JW163	JW12(pJW23)	This study
JW164	JW56(pJW23)	This study
JW165	JW29(pJW23)	This study
JW166	JW63(pJW23)	This study
JW167	JW12(pJW22)	This study
JW168	JW56(pJW22)	This study
JW169	JW29(pJW22)	This study
JW170	JW63(pJW22)	This study
JW175	JW12(pJW19)	This study
JW176	JW56(pJW19)	This study
JW177	JW29(pJW19)	This study
JW178	JW63(pJW19)	This study
JW179	JW1(pJW18)	This study
JW180	JW2(pJW18)	This study
JW181	JW3(pJW18)	This study
JW194	JW1(pJW23)	This study
JW195	JW2(pJW23)	This study
JW196	JW3(pJW23)	This study

Table 2.2. Plasmids

Plasmid	Antibiotic Marker	Relevant Characterisitcs[1]	Reference or Source
pBAD18	Amp	Cloning vector containing *araBAD* promoter	Guzman *et al.*(1995)
pUC18	Amp	High copy cloning vector	New England Biolabs
pUC19	Amp	High copy cloning vector	New England Biolabs
pCS26	Kan	Low copy *luxABCDE* reporter plasmid	Beeston and Surette. (2002)
pNLP10	Kan	pCS26 with expanded MCS	N.L. Price and T.L Raivio, unpublished
pND18	Amp	pBAD18 derivative with *nlpE* cloned into the *Sal*I and *Hind*III sites	Danese *et al.* (1995)
p34S-Cm2	Amp, Cam	Carries *cat* gene with *Eco*R1 site removed	Dennis and Zylstra (1998)
p34S-Tc	Amp, Tc	Carries *tetR* gene cassette	Dennis and Zylstra (1998)
pROX	Amp	pBAD18 derivative with *cpxR* cloned in *Bam*H1 and *Eco*R1 sites	T.L. Raivio, unpublished
pJLM164	Amp, Kn	pRS551derived LEE1::*lacZ* fusion	V. Sperandio; Mellies *et al.*, 1999
pHSG415	Amp, Kn, Cam	gene replacement vector	White *et al.* (1999)
pJW2	Kn	pNLP10 derivative carrying *Eco*RI *Bam*H1 digested *cpxR* promoter PCR fragment	This study
pJW3	Kn	pNLP10 derivative with *Bam*H1 *Eco*R1 fragment containing *LEE1* promoter from pJLM164 cloned into *BamH1* and *Eco*R1 sites	This study

Plasmid	Antibiotic Marker	Relevant Characterisitcs[1]	Reference or Source
pJW5	Kn	pNLP10 derivative carrying *Bam*HI *Eco*RI digested *LEE3* promoter PCR fragment	This study
pJW6	Kn	pNLP10 derivative carrying *Eco*RI *Kpn*I digested *perA* promoter PCR fragment	This study
pJW8	Kn	pNLP10 derivative carrying *Eco*RI *Bam*HI digested *LEE4* promoter PCR fragment	This study
pJW9	Amp	pUC19 derivative with 2kb portion of putative EPEC *hsdR* gene cloned into *Eco*R1 and *Bam*H1 sites	This study
pJW10	Amp, Cam	pJW9 derivative with *SmaI* digested *cat* cassette from p34S-Cm2 cloned into *hsdR StuI* site	This study
pJW12	Kn	pNLP10 derivative carrying *Eco*RI *Kpn*I digested *bfpA* promoter PCR fragment	This study
pJW13	Kn	pNLP10 derivative carrying *Eco*RI *Bam*HI digested *LEE2* promoter PCR fragment	This study
pJW15	Kn	pNLP10 derivative with pSC101 origin removed and p15 origin cloned into *Pac*I sites	This study
pJW17	Kn	pJW15 derivative with *Bam*H1 *Eco*R1 fragment containing *LEE1* promoter from pJLM164 cloned into *Bam*H1 and *Eco*R1 sites	This study
pJW18	Kn	pJW15 derivative with *Bam*H1 *Eco*R1 digested *LEE2* promoter PCR fragment	This study
pJW19	Kn	pJW15 derivative with *Bam*H1 *Eco*R1 digested *LEE3* promoter PCR fragment	This study
pJW20	Kn	pJW15 derivative with *Bam*H1 *Eco*R1 digested *LEE4* promoter PCR product	This study

Plasmid	Antibiotic Marker	Relevant Characterisites[1]	Reference or Source
pJW22	Kn	pJW15 derivative with *Bam*H1 *Eco*R1 digested *perA* promoter PCR fragment	This study
pJW23	Kn	pJW15 derivative with *Bam*H1 *Eco*R1 digested *bfpA* promoter PCR fragment	This study
pJW24	Kn	pJW15 derivative with *Bam*H1 *Eco*R1 digested *cpxR* promoter PCR fragment	This study
pJW26	Amp, Tc	pJW9 derivative with *Sma*I cut *tetR* gene from p34S-Tc cloned into *hsdR Stu*I site	This study

1. The primers used to derive PCR fragments can be found in Table 2.3.

were made by mixing equivalent amounts of overnight cultures in LB with ice cold 10% glycerol and storing at -80 °C.

2.1.5 Tissue culture conditions

Hela cells (ATCC CCL2) were grown under 5% CO_2 at 37°C in minimal essential media (MEM) (Gibco, catalogue number 11095) with 10% (vol/vol) fetal bovine serum.

2.2 DNA Isolation, transduction and transformation

2.2.1 Preparation of *E. coli* competent cells

A single colony was used to inoculate 2ml of LB medium. These cultures were grown at 37 °C overnight. This 2 ml culture was then diluted 1:100 into 200ml of LB and incubated for two hours at 37°C and 225 revolutions per minute (rpm). This culture was centrifuged in 50 ml conical polyethylene tubes (Corning) at 4°C(6500 x g, 10 minutes) and washed with 1 X, 1/2 X, and 1/100 X volumes of ice cold 10% glycerol, where X is the original culture volume. The cells were then resuspended in a 1/500 X volume of ice cold 10% glycerol and 50 µl aliquots were used immediately for electroporation or stored at -80 °C.

2.2.2 Preparation of restriction alleviated EPEC competent cells

An overnight culture was prepared from a single colony as above. This culture was diluted 1:50 into 5ml of fresh media and grown to an optical density (measured at 600nm) of between 0.4 and 0.6. At this time the cells were sedimented by centrifugation at room temperature (6500x g, 10 minutes), the pellet was resuspended in 200 µl of LB, transferred to an Eppendorf tube (Rose) and incubated in a thermocycler (Techne) set to 50°C for thirty minutes. The lid heating function was inactivated to prevent overheating the cells and every ten minutes the cells were mixed by inversion. The cells were then incubated on ice for 2 minutes, sedimented by centrifugation (16 000 x g, 1 minute), the supernatant was removed and the cells were resuspended in 1 ml of ice cold 1mM MOPS-20% glycerol. The cells were sedimented again through centrifugation and resuspended in 500 µl of ice cold 10% glycerol. This procedure was repeated one more time before resuspending the cells in 50 µl of ice cold 10% glycerol. The cells were either used immediately for electroporation or stored at -80 °C.

2.2.3 Electroporation of *E. coli* strains

A 50 ml aliquot of electrocompetent cells was thawed on ice, then 1 to 5 µl of DNA in a low ionic strength buffer was added and the solution was gently mixed and incubated on ice for one minute. The mixture was transferred to a 1mm Gene Pulse cuvette (Bio Rad). The cells were placed in a Micro Pulser electroporation apparatus and the "Ec1" setting was used to electroporate the cells. One milliliter of LB was then added to the cuvette and a Pasteur pipette was used to resuspend the cells and transfer them to a 17 x 100mm test tube. The cells were then allowed to recover at 37°C for one hour, shaking at 225 rpm, before they were plated on selective medium.

2.2.4 Chemical transformation of *E. coli* strains

Commercially obtained TOP 10 chemically competent *E. coli* (Invitrogen) were transformed according to the manufacturer's instructions.

2.2.5 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. For low copy plasmids, 5ml of culture was used and the volumes of all kit components were doubled.

2.2.6 P1*vir* Transduction

P1*vir* transductions were performed as described by Silhavy *et al.* (1984).

2.3 DNA Analysis and Purification

2.3.1 Digestion and cloning of DNA

Restriction endonuclease digestions of plasmid DNA were carried out according to the manufacturer's protocol (Invitrogen or Roche). If restriction site-containing PCR products were used as template, the PCR reaction components were removed using a QIAquick PCR Purification Kit (Qiagen) as described by the manufacturer. When required, non-compatible overhangs were digested using the Klenow fragment (Invitrogen) as advised by the manufacturer. After digestion, fragments were purified on an agarose gel as described in section 2.3.4. Ligations were performed using an insert to vector molar ratio of approximately 4:1 for cohesive overhang ligations and approximately 10:1 for blunt end ligations. Cohesive overhang ligation reactions were performed overnight at 16°C while blunt end ligations were performed at room temperature for 4 hours. Ligations were carried out according to the manufacturer's (New England Biolabs or Roche) specifications.

2.3.2 Polymerase chain reaction (PCR)

PCR was used to generate inserts for cloning, to ascertain whether or not transformants carried recombinant plasmids, and to verify chromosomal insertional inactivation mutations. Oligonucleotide primers and their experimental purpose are listed in Table 2.3.

Amplification was performed in 50 µl volumes in 0.6 ml tubes (Rose Scientific). The reactions were performed using *Taq* DNA polymerase reaction buffer (Invitrogen) and were amplified in either a Mastercycler Gradient thermocycler (Eppendorf) or a Genius thermocycler (Techne). Most reactions contained 1.5mM $MgCl_2$, 1X Reaction Buffer, 0.20 mM dNTPs, 100 pmol of each oligonucleotide primer and 1 µl of Taq polymerase (gift from M.A. Pickard, University of Alberta). The reactions used 10ng of plasmid DNA or a bacterial colony lysed by a 99°C heating step as template. To increase reaction specificity 1 M betaine (Sigma) and/or 5% DMSO (Sigma) were used. If difficulty was encountered in amplifying a particular target a magnesium gradient of 1.5 mM to 5 mM and a temperature gradient from 45°C to 65°C were employed along with betaine and DMSO.

Reactions consisted of an initial 5 minute denaturation at 99°C followed by 32 cycles of a 45 second denaturation at 95°C, 45 second primer annealing at 57°C, and 45

Table 2.3. Oligonucleotide primers

Primer	Sequence(5'-3')	Region of Homology	Use
ERI cpx UP	CGGAATTCCGGCAGCGGTAACTATGCGC	218 bp region containing *cpxR* and *cpxP* promoters	Construction of *cpxR* and *cpxP* promoter fusion vectors
BHI cpx DN	CGGGATCCCTGGCTCTCGACTGAAGGG	218 bp region containing *cpxR* and *cpxP* promoters	Construction of *cpxR* and *cpxP* promoter fusion vectors
5' rpoD	GCCGAAGCAGTTTGACTACC	*rpoD* DNA sequence	RT-PCR control
3' rpoD	CTTCACCGATGGACATACGA	*rpoD* DNA sequence	RT-PCR control
degP-F	GCCGCGTAATTTCCAGCAGTT	*degP* DNA sequence	RT-PCR control
degP-R	GCCCCAGCGCAGAGACAATC	*degP* DNA sequence	RT-PCR control
AJG169	TAATCATGCTCGGTAACGAT	*escT* DNA sequence	RT-PCR
AJG 170	TGTATACTCAGGCCGCTGGG	*escT* DNA sequence	RT-PCR
escC-F	TAGAACCTTGCCCTGCATTT	*escC* DNA sequence	RT-PCR
escC-R	CCGCAGATCCACGTCTTAAT	*escC* DNA sequence	RT-PCR
escV-F	TGCTTTAGTTGCCCAGATCC	*escV* DNA sequence	RT-PCR
escV-R	CCAATGTAGGAAAGCCAGGA	*escV* DNA sequence	RT-PCR

Primer	Sequence(5'-3')	Region of Homology	Use
espA-F	CATGGATACATCCAACTACAGC	*espA* DNA sequence	RT-PCR
espA-R	TTGGGCAGTGGTTGACTCCTT	*espA* DNA sequence	RT-PCR
tir-F	GCTGCATACCGTTACGTCAT	*tir* DNA sequence	RT-PCR
tir-R	TGCGTATTGAGAATATCAAG	*tir* DNA sequence	RT-PCR
perA-L	CGAACCTCAATGAAATGCAA	*perA* DNA sequence	RT-PCR
perA-R	CTGACATCGCCTAGTTTCCAG	*perA* DNA sequence	RT-PCR
ER1 LEE2/3-F	CGGAATTCTATGCGATGCGATGATTAGG	860 bp region containing *LEE3* promoter	Construction of *LEE3* promoter fusion vector
BH1 LEE2/3-R	ATGGATCCATCCTGCGAACTCGTTCAAT	860 bp region containing *LEE3* promoter	Construction of *LEE3* promoter fusion vector
proLEE4B-F	GGGAATTCTCACGCTAGCCAGGATAAGA	560 bp region containing *LEE4* promoter	Construction of *LEE4* promoter fusion vector
proLEE4B-R	ATGGATCCAAACAGATGCGGTGTTTTGA	560 bp region containing *LEE4* promoter	Construction of *LEE4* promoter fusion vector

Primer	**Sequence(5'-3')**	**Region of Homology**	**Use**
ERI pro tir-F	CGGAATTCTGAACAGAAATTTGGTGGTTTG	767 bp region containing *tir* promoter	Construction of *tir* promoter fusion vector
BHI tir-R	ATGGATCCGGTGCAGGTGGAATTAAAGC	767 bp region containing *tir* promoter	Construction of *tir* promoter fusion vector
proPER-L	CGGAATTCTACTCACTTAGCCGCGTGTC	500 bp region containing *perA* promoter	Construction of *perA* promoter fusion vector
proPER-R2	GGGGTACCTTAACAATAACGCTAAATTCTCCTC	region containing *perA* promoter	Construction of *perA* promoter fusion vector
p15ori-F	GCTTAATTAACCTCCTGTTCAGCTACTGACG	cloning p15 origin of pACYC184	replacement of pSC101 origin with p15 origin in pNLP10
p15ori-R	GCTTAATTAACGATGATAAGCTGTCAAACATGA	cloning p15 origin of pACYC184	replacement of pSC101 origin with p15 origin in pNLP10
BH1 LEE2/3-F	ATGGATCCTATGCGATGCGATGATTAGG	860 bp region containing *LEE2* promoter	Construction of *LEE2* promoter fusion vector

Primer	Sequence(5'-3')	Region of Homology	Use
ER1 LEE2/3-R	CGGAATTCATCCTGCGAACTCGTTCAAT	860 bp region containing *LEE2* promoter	Construction of *LEE2* promoter fusion vector
hsdR-L2	CGGGATCCGTTCGGGAACAGCTTCAGAG	2KB region containing putative EPEC *hsdR* gene	Cloning part of putative EPEC *hsdR*
hsdR-R	CGGAATTCCAAACAACGTTATGGCATCG	2KB region containing putative EPEC *hsdR* gene	Cloning part of putative EPEC *hsdR*
hsdR short-F	ATCTGGCACACTACCGGTTC	200bp region of putative EPEC	Insertional inactivation of putative *hsdR* gene using PCR product
hsdR short-R	ATACAGGCAGGTCGCTTTCT	200bp region of putative EPEC	Insertional inactivation of putative *hsdR* gene using PCR product

Underlined sequence indicates restriction site introduced onto end of primer second extension at 72°C. Any exceptions to this PCR protocol are indicated in the results section.

2.3.3 DNA analysis using agarose gel electrophoresis

DNA fragments were size fractionated by electrophoresis at 120V on a 1% agarose, 1 X TAE (40 mM Tris-acetate, 1mM EDTA) gel. A 1 kb ladder (Invitrogen) was used as a molecular weight marker. To each sample 1/6 volume loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) was added to track DNA progression through the gel. Gels were then stained in 1 X TAE containing ethidium bromide, bands were visualized on a UV transilluminator, and images were taken and processed using a FluorChem 5500 imaging system (Alpha Innotech).

2.3.4 Purification of DNA from agarose gels

DNA fragments were size fractionated as described above and the fragment(s) of interest were excised and removed into a 1.5 ml microcentrifuge tube (Rose scientific). The DNA was isolated from the agarose gel using a GeneClean kit (Q Biogene) according to the manufacturer's instructions.

2.4 RNA Analysis

2.4.1 Isolation and quantification of RNA

Total RNA was isolated from mid log phase cells using the Masterpure RNA purification kit (Epicentre). The manufacturer's protocol was followed except the cell lysate was treated with 150 μg of proteinase K and the isolated nucleic acid was treated with 30 units of DnaseI for 90 minutes. RNA was quantified through absorbance at wavelengths of 280nm and 260nm using a spectrophotometer (Pharmacia) and concentration was determined through the formula µg RNA= $(A_{280})(40mg/1.0\ A_{280})$. Concentration was verified by using 0.1 µg of RNA in an RT-PCR reaction amplifying the *rpoD* transcript, a non-Cpx regulated housekeeping gene. The absence of contaminating genomic DNA was verified by performing a PCR reaction in the absence of reverse transcriptase.

2.4.2 Reverse-transcriptase polymerase chain reaction (RT-PCR)

All RT-PCR reactions were performed with the Superscript One-Step RT-PCR kit with Platinum *Taq*, according to the manufacturer's instructions (Invitrogen Life Technologies). All RT-PCR reactions used 1 µl each of a 100mM stock of the forward and reverse primers. The cDNA synthesis reaction was performed by incubating 0.1 mg of total RNA at 55°C for 30 minutes in the presence of reverse transcriptase. The reverse transcriptase was inactivated by heating at 94°C for 2 minutes. Thirty-five PCR cycles were then performed (94°C for 15 s, 55°C for 30 s, and 72°C for 1 min per cycle) with a final extension at 72 °C for 10 minutes. The primers and targets in the RT-PCR were: per-F and per-R (*perA*), AJG169 and AJG170 (*escT*), escC-F and escC-R (*escC*), escV-F and escV-R (*escV*), espA-F and espA-R (*espA*), tir-F and tir-R (*tir*), degP-F and degP-R (*degP*), and 5'rpoD and 3'rpoD (*rpoD*).

2.4.3 Image densitometry analysis of RT-PCR products

After the RT-PCR products were size fractionated on a 1% TAE-agarose gel and stained with ethidium bromide, the gel was photographed using the FluorChem 5500 imaging system (Alpha Innotech). The resulting TIF image was opened in NIH image (available http://rsb.info.nih.gov/nih-image/Default.html), the background was subtracted, and the Measure function was used to obtain image density in pixels per area.

2.5 Protein isolation and analysis

2.5.1 SDS-PAGE of secreted proteins and immunoblotting

Isolated bacterial colonies were inoculated into 5ml of LB and grown overnight at 37°C. The following day the bacteria were diluted 1:50 in 25ml of DMEM containing 0.2% arabinose and grown to the appropriate optical density. Secreted proteins were isolated as described by Kenny *et al.* (1997). Briefly, 1.5 ml of bacteria were transferred to a microcentrifuge tube (Rose) and centrifuged (16 000 x g, 5 minutes). The secreted proteins were precipitated by transferring 1.35ml of supernatant to a fresh microfuge tube containing 150 µl of trichloroacetic acid and incubated for 60 minutes on ice. The precipitated proteins were sedimented by centrifugation at 4°C (16 000 x g, 10 minutes). Samples were then resuspended in 20µl of loading buffer (62.5 mM Tris [pH 6.8], 10% glycerol, 5% β-mercaptoethanol, 3% SDS, 0.1% bromophenol blue) containing 0.1M Tris [pH 8], boiled for 5 minutes, and then resolved on a 12% polyacrylamide gel using a SDS-PAGE minigel system (BioRad) as described by Laemmli (1970). Proteins from the gel were then electroblotted for one hour at 100V onto a nitrocellulose membrane as described by Towbin *et al.* (1970) and visualized through staining in Sypro Ruby Protein

Blot Stain (Bio-Rad) according to the manufacturer's protocol. The Sypro stained blot was then blocked by incubation in 3% MS (3% powdered milk, 0.9% NaCl, 10 mM Tris-Cl [pH 7.5]) for one hour at room temperature as described by Raivio *et al.* (1999). The membranes were then immunoblotted using the primary antibody for one hour. Anti-EspB and anti-Tir antibody were diluted 1:200 and anti-β-galactosidase antibody was diluted 1:1000 in 3% MS. The blots were then washed by shaking vigorously in WS (0.4% Tween, 0.9% NaCl, 10 mM Tris-Cl [pH 7.5]) at room temperature five times for 10 minutes each wash. The blot was then incubated with the secondary antibody for one hour shaking at room temperature. The secondary antisera used was rabbit anti-mouse IgG conjugated to alkaline phosphatase (Sigma) and was diluted 1:3000 in 3% MS. After another series of washes antibody binding was detected using ImmunStar AP Substrate (BioRad) and visualized according to the manufacturer's protocol.

2.5.2 SDS-PAGE of cellular proteins

Bacteria were grown as described in section 2.5.1. The cell pellets from the 1.5 ml samples of culture that were centrifuged to isolate secreted proteins were saved. 50 µl of 2X SDS-PAGE loading buffer (125 mM Tris [pH 6.8], 20% glycerol, 10% β-mercaptoethanol, 6% SDS, 0.2% bromophenol blue) was added and the samples were immersed in a boiling water bath for 5 minutes to denature the proteins. The proteins were then resolved on a 12% polyacrylamide gel as described by Laemmli (1970). After electroblotting to the nitrocellulose membrane, blocking and immunoblotting were performed as described in section 2.5.1.

2.5.3 Enzyme linked immunosorbent assay (ELISA)

Bacteria were grown as in the SDS-PAGE experiments, except that culture volumes of 5ml were used. At appropriate time points 500 µl of bacteria were removed and centrifuged (16 000 x g, 5 minutes) and the supernatant was transferred to a fresh microfuge tube. After all liquid was removed, the pellet was lysed by the addition of 50 µl of 0.1% SDS. Tir ELISAs were performed as described by Kenny *et al.* (1997b). Briefly, 50µl of supernatant was used to coat the wells of a microtitre plate (Corning, catalogue number 3590) and incubated overnight at 4°C. EspB ELISAs were performed in the same manner except a 1:10 dilution of the supernatant in PBS was performed before coating the wells of the plate. β-galactosidase ELISAs were performed by diluting the cell lysate 1:100 in PBS and coating the wells with 50 µl of the dilution then incubated overnight at 4°C as well. The following day the plates were washed three times with distilled water, followed by a one hour blocking at 37°C with 100 µl 0.1% Tween-PBS. The plates were then washed as before and incubated for 1 hour at 37°C with 100 µl of a primary antibody dilution. Mouse anti-EspB and Tir antibodies were diluted 1:200 in 0.1% Tween-PBS while mouse anti-β-galactosidase antibody was diluted 1:1000. The plates were then washed as before and incubated for 1 hour at 37°C with 100 µl of a 1:3000 dilution of peroxidase conjugated affinity purified goat anti-mouse IgG secondary antisera (Sigma) in 0.1% Tween-PBS. The plates were again washed three times with distilled water and 200 µl of developing solution (30 mg of *o*-phenylenediamine dihydrochloride [Sigma] in 30 ml of 0.1 M citric acid [pH 4.4]) was added. The reaction was allowed to proceed for ten minutes before quenching with 100 µl of 3N sulfuric acid. The plates were read at 490nm.

2.5.4 Hemolysis assay

The hemolysis assay was performed as previously described. (Warawa *et al.*, 1999; Blocker et al., 1999). Bacteria were grown overnight in LB, and either diluted 1:50 into fresh media and grown shaking at 37°C in 5 ml glass tubes or diluted 1:20 into fresh media and grown statically in 2ml volumes in 6 well plates (Corning). At an early log phase OD_{600} (0.2-0.5) 0.5 ml of culture was removed and added to a 1.5 ml microcentrifuge tube (Rose) containing an equal volume of a 4% sheep red blood cell-DMEM solution. The bacteria were sedimented onto the blood cells by centrifugation (1500 x g, 2 minutes) and incubated at 37°C for one hour. For background, the 4% RBC-DMEM solution was incubated with DMEM only (B) and for total hemolysis the blood cells were lysed by the addition of 10 µl of 10% SDS (T). Hemolysis was quantified by photometrically measuring hemoglobin release at 543nm. The percent total hemolysis (P) was calculated by the formula $P=(X-B)/(T-B) \times 100$, where X is the absorbance at 543nm of the sample.

2.6 Reporter assays

2.6.1 β-galactosidase reporter assay

Three individual colonies from each strain to be assayed were grown overnight in independent 5 ml LB cultures at 37°C, then diluted 1:50 into 200 ml of LB. After inoculation five 2ml samples were taken at 2, 4, 6, and 8 hours. The samples were sedimented by centrifugation (3220 x g) for 10 minutes then resuspended in 2ml of 1X Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM b-mercaptoethanol). For each sample 250 µl were removed to measure the absorbance at

600nm in a 96 well flat bottom microtitre dish (Fisher). To the remaining sample one drop of chloroform and two drops of 1% SDS were added and cells were lysed by vortexing 30 seconds. Fifty µl of this solution was transferred to a well of a 96-well microtitre dish containing 150 µl of Z-buffer and 50 µl of a freshly made 10mg/ml ONPG solution was then introduced to start the reaction. The absorbance at 420nm was then taken every 5 minutes for 45 minutes. Miller units were then calculated by the formula (A_{420}/OD_{600})(1000) where A_{420} is the slope of the line obtained from graphing absorbance versus time.

2.6.2 Luciferase reporter assay

Five independent colonies were inoculated into 5 ml LB in 16 x 125 mm test tubes and grown overnight shaking at 37°C. The resulting culture was diluted 1:100 in LB or DMEM containing either 0.2% glucose or 0.2% arabinose, plus the appropriate antibiotic selection. The bacteria were grown in either 25 ml volumes in LB or DMEM with 0.2% arabinose or glucose, and after two hours 5 samples of 250 µl were transferred to a 96 well clear bottomed opaque plate (Fisher) and the luminescence, measured in counts per second (cps), and OD_{600} were measured using a Wallac Victor^2 1420 multilabel counter.

2.7 Swim assay

The swim assays were performed as described by Prasad *et al.* (1998). Briefly, bacteria were grown overnight shaking in LB broth at 37°C then diluted 1:100 into LB broth and 2 µl were inoculated into the center of a swim agar plate (10 g/L tryptone, 5g/L

NaCl, 0.1g/L thiamine, 3.5g/L agarose). The plates were grown for 22 hours at 37°C, then photographed using the FluorChem 5500 imaging system (Alpha Innotech).

2.8 Pedestal Formation Assay

Coverslips were washed in 70% EtOH/1% HCl in a petri dish, then rinsed 5 times in dH_20, rinsed once in 70% EtOH and allowed to dry. Hela cells were plated on the coverslips in 24 well plates at a concentration of approximately $7x10^4$ cells/well and allowed to grow overnight at 37°C standing in 5% CO_2. The Hela cells were infected the following day by diluting an overnight culture of EPEC 1:200 in 3ml of either glucose free DMEM (Gibco) or MEM/10% FCS, aspirating the media from each well and then adding 1 ml of bacteria to each well . The bacteria were allowed to grow for 3 or 4 hours at 37°C under a 5% CO_2 atmosphere. A paraformaldehyde solution was made by heating a 1ml aliquot of 25% paraformaldehyde to get it in solution and adding 1ml of 10X PBS, 5ml of 20% HCl and bringing the volume up to 10ml with dH_20. The cells were then washed three times with 37°C PBS and fixed with 200 µl of 2.5% paraformaldehyde [pH 7.4] for 10 minutes at 37°C. The cells were washed three times with PBS over a period of 45 minutes. The cells were permeabilized through a 5 minute wash with 200 µl of PBS/0.1% Triton X-100 at room temperature. The permeabilizing buffer was then removed through aspiration and the cover slips were centered in the well using the tip of the aspirator. Each slip had 15 µl of primary antibody added and was incubated at 37°C in 5% CO_2 for one hour. The primary antibody solution contained mouse anti-Tir antibody diluted 1:100 in PBS/0.1% Triton X-100/ 10% normal goat serum, and rabbit anti-EPEC antibody diluted 1:300. After three ten minute washes with PBS/0.1% Triton

X-100 the buffer was removed, the coverslip centered again and then 15 µl of secondary antibody solution was added and the slips were incubated for 30 minutes in a 37°C, 5% CO_2 incubator. The secondary antibody solution contained anti-mouse antibody conjugated to Cy3 diluted 1:600, anti-rabbit antibody conjugated to the fluorescent dye Alexa 350 diluted 1:200, and phalloidin conjugated to the fluorescent dye Alexa 488 diluted 1:200. Three more ten minute washes with PBS/0.1% Triton X-100 were then performed and the coverslip was mounted onto a glass slide with 3-5 µl of Mowiol (Sigma) and polymerized for 30 minutes. Mowiol is made by mixing 3 grams of glycerol, 1.2 grams of Mowiol and 3ml of dH_2O, incubating at room temperature for two hours, then adding 6 ml of 0.2M Tris pH 8.5. After a 53 °C overnight incubation the solution was spun for 20 minutes at 2500 x g and frozen in 1 ml aliquots. The slides were then examined under a fluorescent phase-contrast microscope. A representative image from each slide was photographed in each channel and the images were overlaid using OpenLab software (Improvision).

Alternatively, when bacteria were required to be grown shaking, an overnight culture was diluted 1:100 into glucose free DMEM and grown to the desired OD_{600} at 37°C shaking at 225 rpm. The media was aspirated from the Hela cell wells and 1 ml of bacteria were added. The bacteria were then centrifuged for 4 minutes at 1000 x g to initiate contact between the bacteria and the Hela cells. The bacteria and cells were then coincubated for 40 minutes at 37°C in 5% CO_2, then the cells were fixed, permeablilized and labeled as described above.

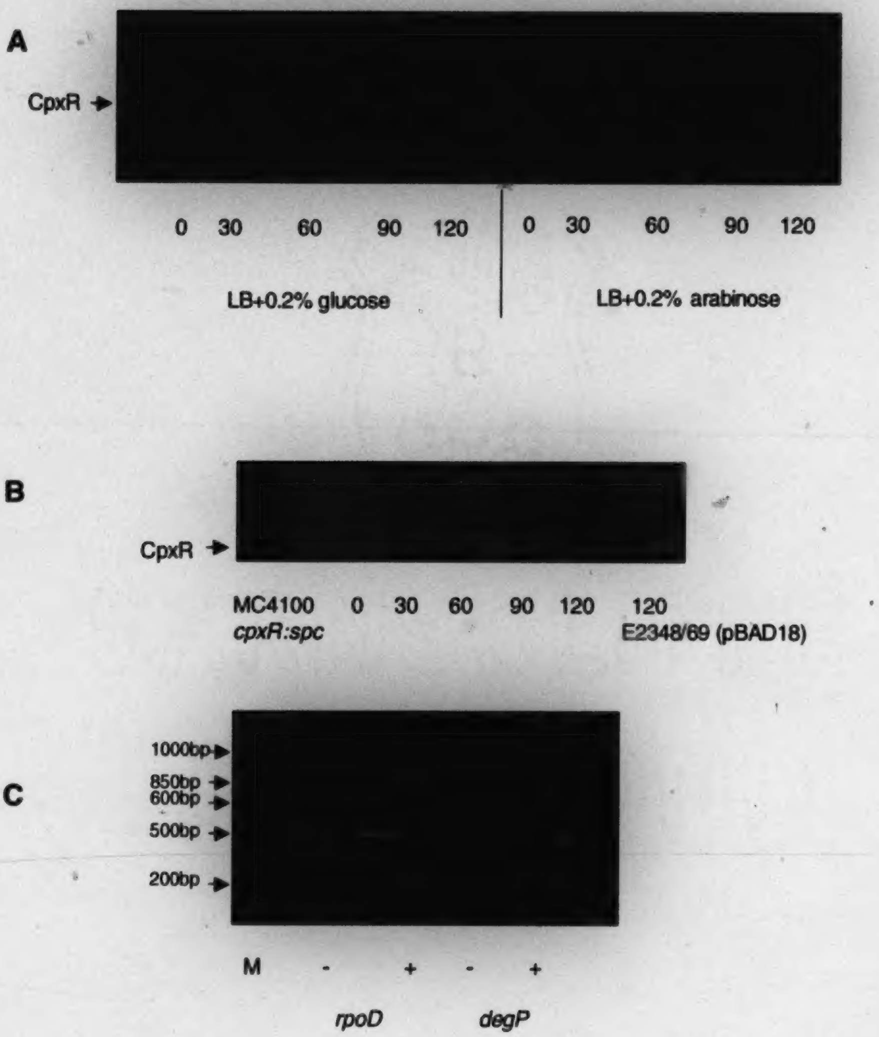
3. The effect of the Cpx response on EPEC virulence determinant secretion

3.1 Analysis of EPEC secreted virulence proteins

3.1.1 Hemolysis

In order to study the Cpx response in EPEC, methods of activating the Cpx pathway in EPEC had to be developed. In MC4100, the arabinose-inducible expression vector pBAD18 has been used to create the *nlpE* overexpression vector pND18 (Danese *et al.*, 1995) and the *cpxR* overexpression vector pROX (Raivio, unpublished). Since both are used to activate the Cpx pathway, these constructs were transformed into the EPEC strains E2348/69 and E2348/69 *cpxR:kn*, which contains an insertionally inactivated *cpxR* gene, preventing induction of the Cpx regulon. Initially, experiments were performed to determine if glucose, which represses the arabinose-inducible P_{BAD} promoter on pBAD18, and arabinose effectively regulated production of CpxR and NlpE. Arabinose was demonstrated to be effective at inducing expression from the P_{BAD} promoter as at 90 minutes an increase in CpxR signal was seen in E2348/69 *cpxR:kn* (pROX) cells grown in 0.2% arabinose but not when the same strain was grown in the presence of 0.2% glucose (Figure 3.1A). The α-CpxR antisera is non-specific as three bands are present on the blots shown in Figure 3.1. The bottom band is CpxR as no band is present in the MC4100 strain TR51, which does not produce CpxR due to an insertional inactivation of the gene (Figure 3.1B). Similar results were obtained when CpxR levels were examined in E2348/69 carrying the NlpE overexpression vector pND18 (Figure 3.1B). CpxR levels increased to a greater level when E2348/69(pND18) was grown in arabinose, relative to E2348/69 carrying the vector control, pBAD18,

Figure 3.1. Detectable Cpx activation in EPEC occurs 90 minutes after inducing *nlpE* or *cpxR* overexpression. (A) Western blotting using polyclonal antisera directed against CpxR was performed on whole cell lysates of EPEC *cpxR:kn*(pROX). Overnight cultures of E2348/69 *cpxR:kn*(pROX)grown shaking in LB at 37°C were diluted 1:50 into LB containing either 0.2% glucose or arabinose and 1 ml samples were taken every 30 minutes, to 120 minutes. Cell pellets were resuspended in 50 uL of 2X SDS-PAGE loading dye and 20 uL of whole cell lysate was electrophoresed on 12 % SDS-PAGE gels and subjected to immunoblotting with anti-CpxR anti-sera. Two unknown cross-reactive species are observed. (B) Western blotting using polyclonal antisera directed against CpxR was performed on whole cell lysates of an EPEC strain, E2348/69, carrying an *nlpE* overexpression plasmid, pND18. An overnight culture of E2348/69 (pND18) grown shaking in LB at 37°C was diluted 1:50 into LB containing 0.2% arabinose, and a 1ml sample was taken every thirty-minutes, to 120 minutes. A lysate of a nonpathogenic strain of *E. coli,* MC4100 *cpxR:spc*, which does not produce CpxR, was included as a control. An overnight culture of E2348/69 (pBAD18) grown shaking in LB at 37°C was diluted 1:50 into LB containing 0.2% arabinose and grown shaking at 37°C for 120 minutes at which point a 1 ml sample was taken. Cell pellets were resuspended in 50 uL of 2X SDS-PAGE loading dye and 20 uL of whole cell lysate was electrophoresed on 12% SDS-PAGE gels then subjected to immunoblotting with anti-CpxR anti-sera. (C) RT-PCR analysis of *rpoD* and *degP*. RNA (0.1 μg) isolated from mid-log phase cells of E2348/69 *cpxR:kn,* an EPEC strain carrying a null mutation in CpxR, grown in LB, carrying either the vector control, pBAD18 (-), or the *cpxR* overexpression vector, pROX (+), was used as a template for RT-PCR. All RT-PCR reactions consisted of 35 cycles (94°C for 15 s, 55°C for 30 s, and 72°C for 1 min per cycle) with a final extension at 72°C for 10 minutes.. An RT-PCR reaction using primers (5' rpoD, 3' rpoD) amplifying *rpoD,* coding for σ^{70} of RNA polymerase, was used as a loading control, to equalize amounts of RNA present in each reaction tube. After RNA concentrations were equalized, an RT-PCR reaction was performed with primers (degP-F, degP-R) to amplify the *degP* transcript. A 1kb+ ladder (Invitrogen) was used as a marker (M). The products were size fractionated on a 1% agarose gel, and bands were visualized through ethidium bromide staining. Each experiment was repeated two times and a representative result is shown.


A
CpxR
0 30 60 90 120
0 30 60 90 120
LB+0.2% glucose
LB+0.2% arabinose
B
CpxR
MC4100 cpxR:spc
0 30 60 90 120
120
E2348/69 (pBAD18)
C
1000bp
850bp
600bp
500bp
200bp
M - + - +
rpoD
degP

grown in arabinose (Figure 3.1B). Since in this experiment CpxR expression is increased solely as a result of Cpx pathway activation by NlpE overexpression, these data indicate that overexpression of NlpE functions to activate the Cpx regulon in EPEC. Together, these experiments show that arabinose induction from P_{BAD} works in an EPEC strain background and that NlpE serves to activate the Cpx response in EPEC in a similar fashion to that in MC4100.

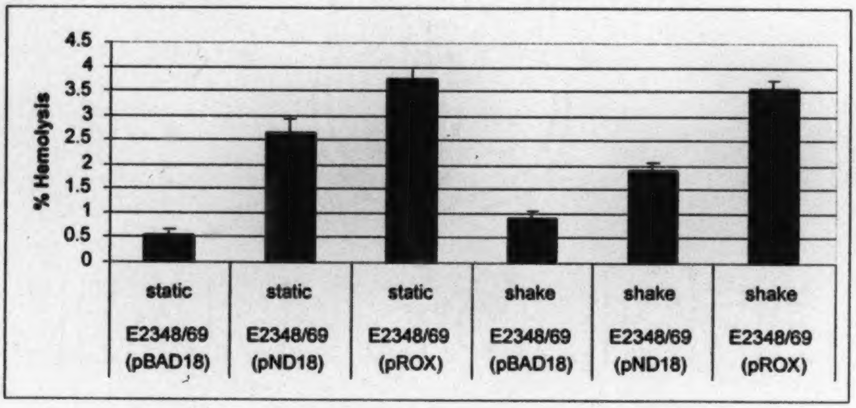
To further show that arabinose induction of CpxR expression from pROX facilitated activation of the Cpx regulon, RT-PCR analysis of *degP* expression was performed. RNA was isolated from EPEC *cpxR:kn* carrying pBAD18 and pROX grown in the presence of arabinose and RT-PCR analysis was performed. To ensure equal amounts of RNA were used, RT-PCR analysis of the *rpoD* gene, encoding the housekeeping sigma factor σ^{70}, was also carried out (Fig 3.1C). The *rpoD* gene is not Cpx-regulated. The RNA was harvested from bacteria subcultured 1:50 into fresh LB-0.2% arabinose and grown to mid-log phase. At this time, amplified *degP* transcript levels increase in E2348/69 *cpxR:kn* carrying the *cpxR* overexpression vector pROX, indicating that the Cpx regulon is activated under these conditions (Figure 3.1C). Cumulatively, these data indicate that *cpxR* and *nlpE* overexpression activate the Cpx pathway in EPEC.

The first logical question that needed to be addressed was whether Cpx activation led to increased activity of virulence factors. The presence of a functional type III secretory system in *Shigella flexneri, Yersinia enterocolitica,* and EPEC has been demonstrated to cause red blood cell (RBC) lysis when the bacteria are in contact with erythrocytes (Blocker *et al.*, 1999; Håkansson *et al.*, 1996; Warawa *et al.*, 1999).

Increased activity of the EPEC type III secretory system results in elevated secretion of EspB and EspD. As these effectors form a pore in the erythrocyte membrane, causing lysis, the effect of Cpx activation on red blood cell hemolysis was examined to determine whether the Cpx system affected type III secretory activity. E2348/69 strains containing the vector control, an *nlpE* overexpression vector and a *cpxR* overexpression vector were grown aerobically in DMEM+ 0.2% arabinose to activate the P_{BAD} promoter and a hemolysis assay was performed. As with protein secretion, EPEC hemolysis is usually observed in bacteria grown statically in CO_2, although secretion can occur in aerobic growth provided the media is supplied with a buffering agent, such as Tris-HCl or HEPES (Kenny *et al.*, 1997; Warawa *et al.*, 1999). The bacteria for the hemolysis assay were grown aerobically in DMEM+0.2% arabinose with 0.1M Tris HCl pH[7.6], shaking at 225 rpm or statically.

Cpx activation caused an increase in hemolysis in cells grown with shaking. *cpxR* overexpression caused hemolysis of 3.57% of the RBCs, *nlpE* overexpression caused lysis of 1.87% and the vector control resulted in a lysis of approximately 1% of the RBCs (Figure 3.2). These data are similar in pattern to the results obtained when the cells were grown statically. E2348/69 containing the *cpxR* overexpression plasmid, pROX, the *nlpE* overexpression plasmid, pND18, and the vector control, pBAD18, caused 3.7%, 2.6%, and 0.56% hemolysis, respectively (Figure 3.2). Static growth appears to cause a moderate increase in hemolysis caused by E2348/69 (pND18) and a decrease in E2348/69 (pBAD18) hemolysis (Figure 3.2). Cpx activation, whether through *cpxR* overexpression or *nlpE* overexpression, increased hemolysis of sheep red blood cells,

Figure 3.2. Sheep red blood cell hemolysis is increased by Cpx activation in both shaking and static growth. Overnight cultures, grown shaking at 37°C in LB, of E2348/69 cells containing the vector control, pBAD18, the *nlpE* overexpression vector, pND18, or the *cpxR* overexpression vector, pROX, were diluted 1:50 in DMEM+0.2% arabinose with 0.1M Tris HCl pH[7.6] and grown to mid-log phase (OD_{600} 0.3-.0.5) shaking at 225 rpm at 37°C or were diluted 1:20 from an overnight culture into 2ml of DMEM+0.2% arabinose with 0.1M Tris HCl pH[7.6] in 6 well plates and grown statically at 37°C to mid-log phase. Bacteria and a 4% red blood cell (RBC)-DMEM solution were mixed 1:1, centrifuged to initiate bacteria-blood cell contact, and incubated for one hour at 37°C. Release of hemoglobin was monitored spectrophotometrically by measuring the absorbance at 543 nm of the supernatant after centrifugation of the bacteria RBC mixture. The percent total hemolysis (P) was calculated by the formula P=(X-B)/(T-B) x 100, where X is the absorbance at 543nm of the sample, B is the absorbance at 543nm of the 4% RBC-DMEM solution, and T is the absorbance at 543nm of the 4% RBC-DMEM solution lysed through the addition of 10 µl of 10% SDS. The error bars represent the standard deviation of three independent assays.


% Hemolysis
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
static
E2348/69
(pBAD18)
static
E2348/69
(pND18)
static
E2348/69
(pROX)
shake
E2348/69
(pBAD18)
shake
E2348/69
(pND18)
shake
E2348/69
(pROX)

3.1.2 ELISA analysis of EPEC secreted proteins

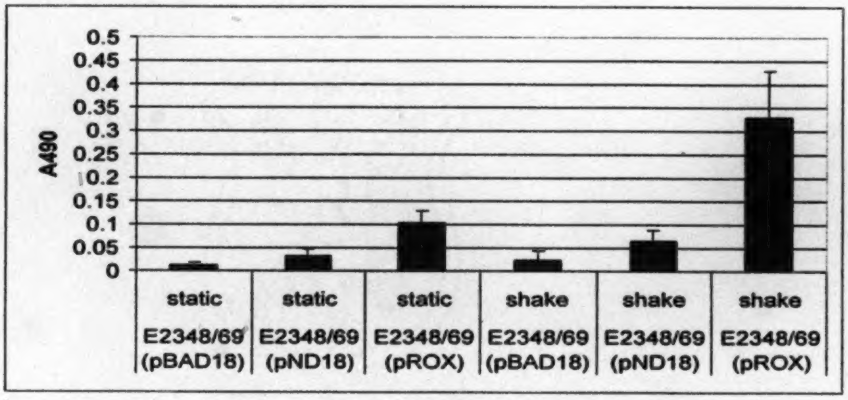
As an increase in hemolysis is indicative of increased type III secretory activity, the effect of Cpx activation on EPEC virulence-determinant secretion was then examined. EspB and Tir enzyme linked immunosorbent assays were developed using the method of Kenny *et al.* (1997b) as they are conducive to high throughput assays and timecourses. Since it uses less supernatant, it can be used to verify virulence protein secretion by bacteria used in other experiments such as hemolysis assays. The experiments described in the majority of the literature analyzing EPEC pathogenicity involve growing the bacteria statically in a 5% CO_2 environment, however, the microaerophilic growth condition is merely required for buffering and 0.1M HEPES or 0.1M Tris-HCl can be utilized to maintain the pH between 6.35 and 7.6, the optimal range for secretion of EspA, EspB, EspC (Kenny *et al.*, 1997). The bacteria were grown as they were in the hemolysis experiment but, to compare the effects of static versus shaking growth, the same strains were grown aerobically, shaking in 5ml tubes, and statically in 6 well plates, in the presence of 0.1M [pH 7.6] Tris-HCl. Supernatant samples and a whole cell lysate of each strain used in the hemolysis assay were collected for ELISA analysis from cultures at an OD_{600} of 0.3-0.5.

There appeared to be no differences in secretion of EspB between E2348/69 strains containing the vector control and an *nlpE* overexpression vector (Figure 3.3). E2348/69 overexpressing *cpxR*, grown shaking, secreted almost three times more EspB compared to static growth (Figure 3.3). This is interesting considering that E2348/69 overexpressing *cpxR* produces similar levels of hemolysis with static and shaking growth. A β-galactosidase ELISA was performed as a control on a 1:100 dilution of the whole cell lysate to ensure that equal amounts of cells were used in the hemolysis and ELISA assays (Figure 3.3). As the amount of β-galactosidase detected was equal, the differences in hemolysis and EspB secretion were not due to differences in the number of cells used. While Cpx activation through NlpE overexpression lead to little, if any, increase in EspB secretion, *cpxR* overexpression resulted in an increase in EspB secretion in both shaking and static cutltures (Figure 3.3). Since CpxR overexpression is a stronger inducer of Cpx-regulated gene expression, these results suggest that EspB secretion is increased only in response to strong stimulation of the Cpx pathway

A 1:10 dilution of the supernatant was required to detect any differences in EspB secretion in E2348/69 strains carrying the vector control and *nlpE* and *cpxR* overexpression plasmids, as failure to do so resulted in a saturation of the colorimetric assay. This indicates that EspB is secreted by EPEC at high levels in the assay conditions employed. In contrast, the Tir ELISA did not require a dilution of the supernatant, indicating that Tir was present at much lower levels. The Tir ELISAs were performed on supernatants from cells grown shaking. This is a condition that usually does not result in maximal Tir secretion (Kenny *et al.*, 1997b). *cpxR* and *nlpE* overexpression caused increased secretion of Tir relative to the vector control. The vector control resulted in a level of secretion in the magnitude of a secretion deficient mutant of E2348/69, CFM 14-2-1 (Figure 3.4). Finally a β-galactosidase ELISA was performed on culture supernatant to ensure that the detected EspB and Tir proteins were due to elevated secretion rather than increased cell lysis, perhaps caused by envelope alterations due to activation of the Cpx response (Figure 3.4). Since all supernatant samples had essentially the same level of β-galactosidase protein, the increased EspB and Tir levels were due to type III

Figure 3.3. EspB secretion is increased by Cpx activation under both static and shaking growth. (A) Overnight cultures of E2348/69 strains carrying the vector control, pBAD18, the *nlpE* overexpression plasmid, pND18, and the *cpxR* overexpression plasmid, pROX, grown shaking in LB at 37°C, were diluted 1:50 into DMEM+0.2% arabinose with 0.1M [pH 7.6] Tris-HCl and grown at 37°C , either statically or shaking at 225 rpm, as in the hemolysis assay. 50 μl of supernatant was removed, diluted 1:10 in PBS, and then 50 μl was used to coat the wells of a protein binding enzyme-linked immunosorbent assay (ELISA) plate. An ELISA was then carried out with EspB antisera. (B) 500 μl of the bacterial culture used in the hemolysis assay and subsequently used to generate the supernatant for the EspB ELISAs, was sedimented, the supernatant removed and the bacteria were lysed through addition of 50 μl of 1% SDS. 50 μl of a 1:100 dilution of the cell lysate in PBS was used to coat the wells of an EIA plate and an ELISA was performed with β-galactosidase antisera. The error bars represent standard deviation of 5 independent samples. The experiment was performed twice and a representative result is shown.

A


A490
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
static E2348/69 (pBAD18)
static E2348/69 (pND18)
static E2348/69 (pROX)
shake E2348/69 (pBAD18)
shake E2348/69 (pND18)
shake E2348/69 (pROX)

B

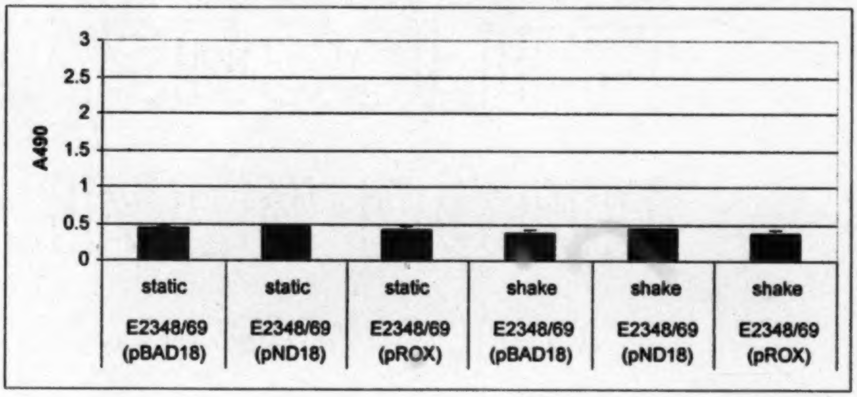
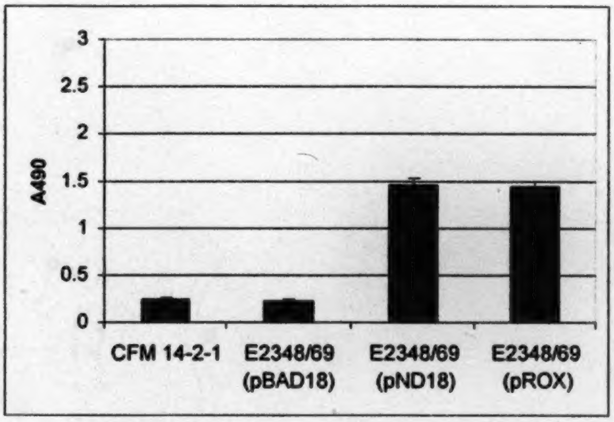
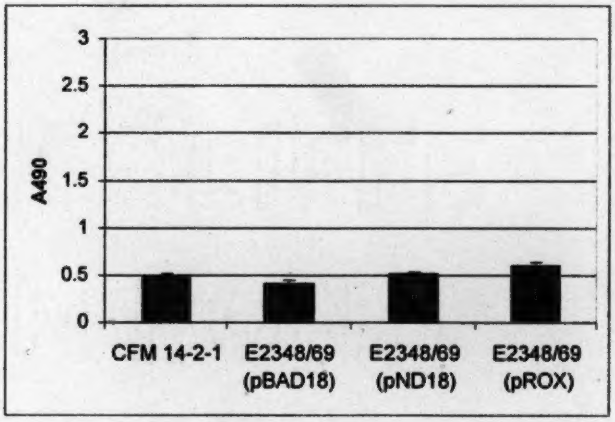

A490
3
2.5
2
1.5
1
0.5
0
static E2348/69 (pBAD18)
static E2348/69 (pND18)
static E2348/69 (pROX)
shake E2348/69 (pBAD18)
shake E2348/69 (pND18)
shake E2348/69 (pROX)

Figure 3.4. Cpx activation increases Tir secretion. (A) Overnight cultures of E2348/69 carrying the vector control, pBAD18, the *nlpE* overexpression vector, pND18, the *cpxR* overexpression vector, pROX, and a type III secretion deficient E2348/69 strain, CFM 14-2-1, grown shaking in LB at 37°C were diluted 1:50 in DMEM+0.2% arabinose with 0.1M [pH 7.6] Tris-HCl, and grown shaking at 225 rpm at 37°C to an OD_{600} of 0.5. 500 µl of bacteria were sedimented by centrifugation, the supernatant was used to coat the wells of a protein binding EIA plate and Tir secretion levels were determined through ELISA with Tir antisera. (B) To ensure equal numbers of bacteria were used to obtain the supernatant, the cell pellet was resuspended in 50 µl of 1% SDS and a 1:100 dilution of the cell lysate in PBS was used to coat the wells of an EIA plate to assay cellular β-galactosidase levels by ELISA. (C) To verify that Cpx activation was not increasing cell lysis, overnight cultures of E2348/69 carrying pBAD18, pND18, and pROX, grown shaking in LB at 37°C, were diluted 1:50 in DMEM+0.2% arabinose and grown shaking at 225 rpm at 37°C to an OD_{600} of 0.5. The bacteria were sedimented by centrifugation, 50 µl of supernatant was used to coat the wells of an EIA plate, and β-galactosidase levels in the supernatant were determined through ELISA. The error bars represent standard deviation of 5 independent samples. The experiment was performed twice and a representative result is shown.

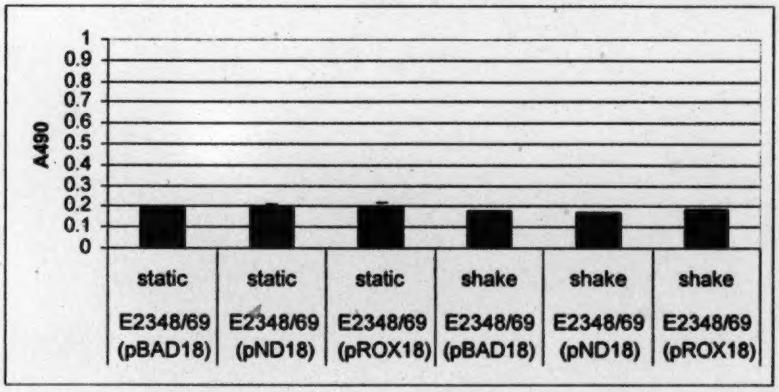
A


3
2.5
2
1.5
1
0.5
0
A490
CFM 14-2-1
E2348/69 (pBAD18)
E2348/69 (pND18)
E2348/69 (pROX)

B


3
2.5
2
1.5
1
0.5
0
A490
CFM 14-2-1
E2348/69 (pBAD18)
E2348/69 (pND18)
E2348/69 (pROX)

C


1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A490
static E2348/69 (pBAD18)
static E2348/69 (pND18)
static E2348/69 (pROX18)
shake E2348/69 (pBAD18)
shake E2348/69 (pND18)
shake E2348/69 (pROX18)

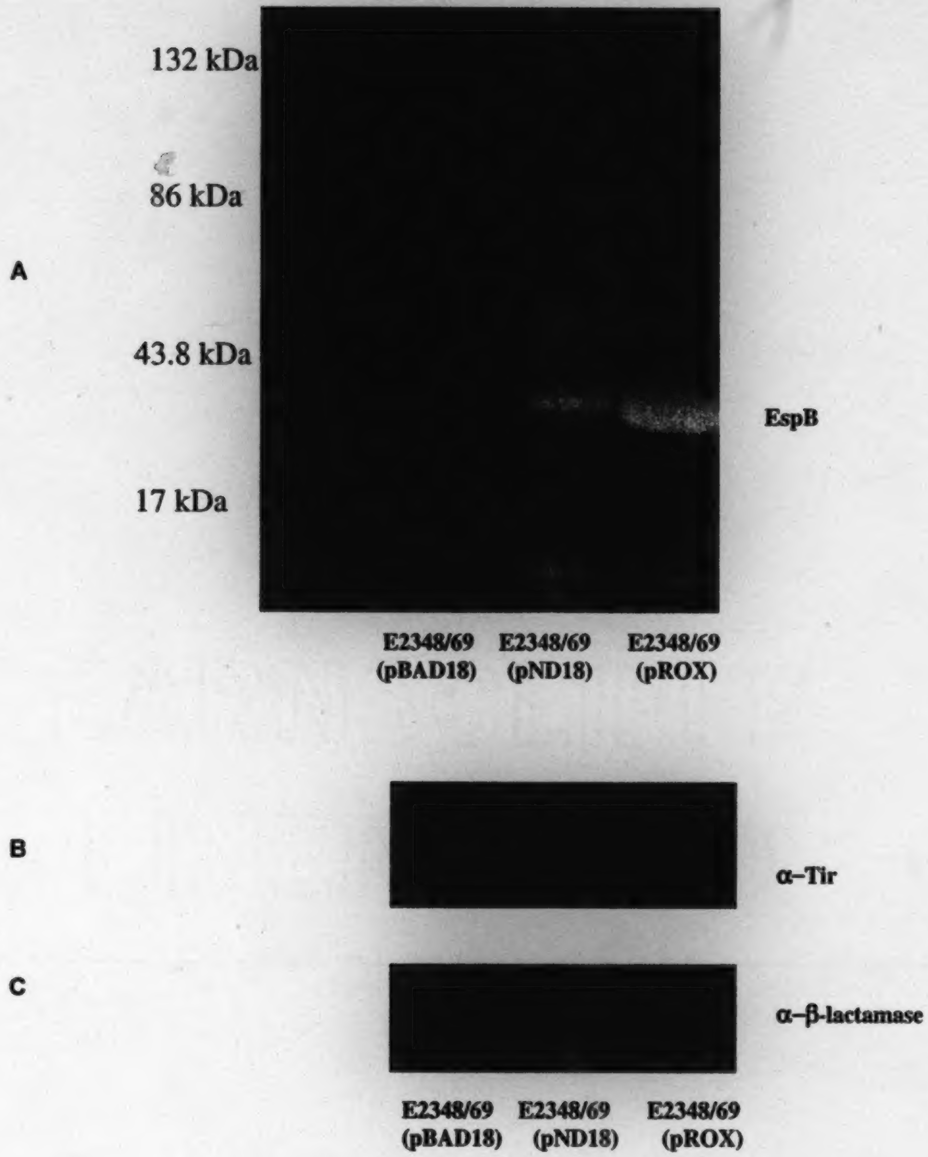
secretion. The ELISA data indicated that Cpx activation, through either *nlpE* or *cpxR* overexpression, increased Tir secretion. Only *cpxR* overexpression produced an increase in EspB secretion detectable by ELISA.

3.1.3 Immunoblot analysis of EPEC secreted proteins

The ELISA data suggested that Cpx activation caused an increase in virulence determinant secretion. To verify this observation immunoblot assays were performed on TCA precipitated supernatants from cultures grown at 37°C, shaking at 225 rpm to an OD_{600} of 0.5, 0.7, and 1.0. After the precipitated secreted proteins were size fractionated on a 12% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane, the blot was stained with Sypro Ruby stain (BioRad). The advantage to using this stain is that it is a highly sensitive fluorescent stain that non-covalently interacts with proteins, thus allowing further analysis of the membrane through immunoblotting. Abundant proteins on the blot can be visualized through UV exposure giving results similar to a Coomassie stained polyacrylamide gel. At an OD_{600} of 0.5, CpxR overexpression increased EspB secretion, but no changes in the E2348/69 strains overexpressing *nlpE*, or carrying the vector control can be seen (data not shown). At an OD_{600} of 0.7, NlpE overexpression produces an observable change in EspB secretion, so this timepoint was shown in Figure 3.5. No significant changes were observed between the OD 0.7 and 1.0 samples. Thus, both NlpE and CpxR overexpression appear to elevate levels of secreted EspB, as measured by Sypro Ruby-stained blots.

The same blot was then immunoblotted using an anti-Tir antibody, as Tir is secreted at low levels, making it difficult to detect through Sypro Ruby staining

Figure 3.5. EPEC secreted proteins are increased by Cpx activation. (A) Overnight cultures of E2348/69 cells containing the vector control, pBAD18, the *nlpE* overexpression vector, pND18, or the *cpxR* overexpression vector, pROX, grown shaking in LB at 37°C, were diluted 1:50 in DMEM+0.2% arabinose and grown shaking at 225 rpm, 37°C, to an OD_{600} of 0.7. 1.5 ml of cells were sedimented through centrifugation and 1.35 ml of supernatant was mixed with 150 µl of trichloroacetic acid to precipitate the supernatant proteins. The proteins were sedimented through centrifugation, resuspended in 20 µl of SDS –PAGE loading buffer containing 0.1M Tris [pH8], resolved through electrophoresis on a 12% SDS-polyacryamide gel, electroblotted onto a nitrocellulose membrane and stained with Sypro Ruby stain. (B) An immunoblot using Tir antisera was performed on the Sypro Ruby stained blot. (C) The bacteria sedimented to obtain the supernatant proteins were lysed in 50 µl of 2X SDS-PAGE sample buffer, then 20 µl of the lysate was resolved on a 12% SDS-polyacrylamide gel and an immunoblot using β-lactamase antisera was performed to ensure that equal numbers of bacteria were sedimented to obtain the supernatant. The experiment was performed twice and a representative result is shown.


132 kDa
86 kDa
A
43.8 kDa
EspB
17 kDa
E2348/69 (pBAD18)
E2348/69 (pND18)
E2348/69 (pROX)
B
α–Tir
C
α–β-lactamase
E2348/69 (pBAD18)
E2348/69 (pND18)
E2348/69 (pROX)

(Figure 3.5). An immunoblot of a whole cell lysate of the bacteria sedimented to obtain the secreted proteins was probed with β-lactamase antisera as a control to ensure that equal amounts of bacteria were used to isolate the secreted proteins (Figure 3.5). When E2348/69 carrying pBAD18 is grown in DMEM+0.2% arabinose, shaking, at 37°C, no EspB protein can be detected by Sypro Ruby staining, demonstrating that the conditions we employ do not normally activate virulence gene secretion (Figure 3.5). However, when *nlpE* or *cpxR* are overexpressed, the EspB protein is secreted and can be detected. Activation of the wildtype Cpx pathway through *nlpE* overexpression results in less EspB secretion than overexpression of the response regulator, *cpxR*, does (Figure 3.5). Similarly, no Tir secretion can be detected by immunoblotting the precipitated, secreted proteins of an E2348/69 strain carrying the vector control, but Tir can be detected in the supernatants of E2348/69 strains overexpressing *nlpE* and *cpxR*. Again CpxR overexpression causes an increased Tir secretion relative to NlpE overexpression (Figure 3.5). Immunoblotting experiments, which are more sensitive than enzyme-linked immunosorbant assays, revealed that Cpx activation through *nlpE* overexpression or *cpxR* overexpression result in an increase in EspB and Tir secretion.

3.1.4 NlpE and EPEC secretion

As the NlpE activating signal is relayed by NlpE to the Cpx system it was expected that ALN88, an E2348/69 *cpxR:kn* mutant strain, would not display an increase in secretion in EspB or Tir secretion when *nlpE* was overexpressed. To test this hypothesis, supernatant proteins were precipitated from ALN88 strains carrying the vector control, pBAD18, the *nlpE* overexpression vector, pND18, and the *cpxR* overexpression vector, pROX, grown shaking at 37°C in DMEM+0.2% arabinose to an OD_{600} of 0.5. The proteins were size fractionated on a 12% SDS-polyacrylamide gel, electroblotted onto a nitrocellulose membrane and the blot was stained with Sypro Ruby stain (BioRad). Surprisingly at an OD_{600} of 0.5, *nlpE* overexpression caused a significant increase in EspB secretion in the absence of the response regulator *cpxR* (Figure 3.6). ALN88(pROX) displayed an increase in EspB secretion relative to the vector control, ALN88(pBAD18), though not as large as the increase caused by *nlpE* overexpression. The Sypro stained blot was then immunoblotted using Tir antisera (Figure 3.6) and an immunoblot of a whole cell lysate of the bacteria sedimented to obtain the secreted proteins was probed with β-lactamase antisera as a loading control (Figure 3.6). In the absence of the response regulator, *cpxR*, *nlpE* overexpression still causes increased EspB and Tir secretion in EPEC through an unknown mechanism.

3.2 Pedestal forming assay

Since Cpx activation resulted in increases in EPEC protein secretion, and this was correlated with increased hemolysis of red blood cells, an assay was performed to determine whether or not Cpx activation also resulted in increased actin pedestal formation when the bacteria were incubated with Hela cells. E2348/69 strains carrying the pBAD18 vector control and the pROX *cpxR* overexpression plasmid were grown shaking at 225 rpm at 37°C in the presence of 0.2% glucose or 0.2% arabinose to an OD_{600} of approximately 0.5. The bacteria were added to the Hela cells and centrifuged for 4 minutes at 1000 x g to initiate contact between the bacteria and Hela cells. After a

Figure 3.6. EspB and Tir secretion are increased by *nlpE* overexpression in the absence of *cpxR*. (A) Overnight cultures of ALN88 (E2348/69 *cpxR:kn)* cells containing the vector control, pBAD18, the *nlpE* overexpression vector, pND18, or the *cpxR* overexpression pROX, grown shaking in LB at 37°C, were diluted 1:50 in DMEM+0.2% arabinose and grown shaking at 225 rpm, 37°C to an OD_{600} of 0.5. 1.5 ml of cells were centrifuged and1.35 ml of supernatant was mixed with 150 µl of trichloroacetic acid to precipitate the supernatant proteins. The proteins were sedimented through centrifugation, resuspended in 20 µl of SDS-PAGE loading buffer with 0.1M Tris [pH8], resolved through electrophoresis on a 12% SDS-polyacryamide gel, electroblotted onto a nitrocellulose membrane and stained with Sypro Ruby stain. (B) An immunoblot using Tir antisera was then performed on the Sypro stained blot. (C) The bacteria sedimented to obtain the supernatant proteins were resuspended in 50 µl of 2x SDS-PAGE sample buffer, 20 µl was resolved on a 12% SDS-polyacrylamide gel and an immunoblot using β-lactamase antisera was performed to ensure that equal numbers of bacteria were used. This experiment was repeated twice and a representative result is shown.

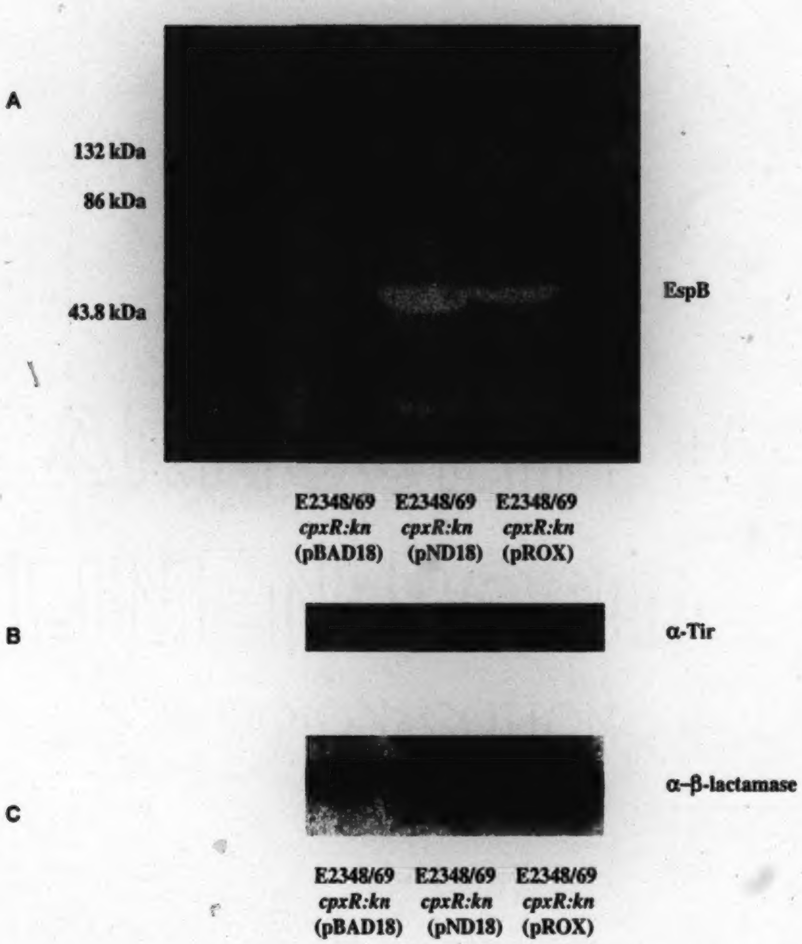

A
132 kDa
86 kDa
43.8 kDa
EspB
E2348/69 *cpxR:kn* (pBAD18)
E2348/69 *cpxR:kn* (pND18)
E2348/69 *cpxR:kn* (pROX)
B
α-Tir
C
α–β-lactamase
E2348/69 *cpxR:kn* (pBAD18)
E2348/69 *cpxR:kn* (pND18)
E2348/69 *cpxR:kn* (pROX)

Figure 3.7. Growth in DMEM+0.2% arabinose appears to inhibit bacterial adherence. Hela cells were seeded onto glass coverslips and grown overnight under 5% CO_2 in MEM/10% FCS. Overnight cultures of EPEC strains carrying either the vector control, pBAD18, or the *cpxR* overexpression vector, pROX, grown shaking in LB, at 37°C, were diluted 1:50 in DMEM+0.2% glucose or 0.2% arabinose and grown shaking at 225 rpm and 37°C to an OD_{600} of 0.5. The media was aspirated from the Hela cells and replaced with 1 ml of bacterial culture which was centrifuged for 4 minutes at 1000 x g to initiate contact between the bacteria and Hela cells. The bacteria and Hela cells were coincubated for forty minutes at 37°C under a 5% CO_2 atmosphere before the media was removed and the cells were fixed with 2.5% paraformaldehyde. The cells were labeled with anti-Tir (red), anti-EPEC (blue), and phalloidin (green), examined through immunofluorescent microscopy and the overlay of the three stains is shown with colocalization producing white foci. The magnification of the image shown is 100X. This experiment was performed twice and a representative result is shown.

E2348/69 (pROX)+glc E2348/69 (pROX)+ ara

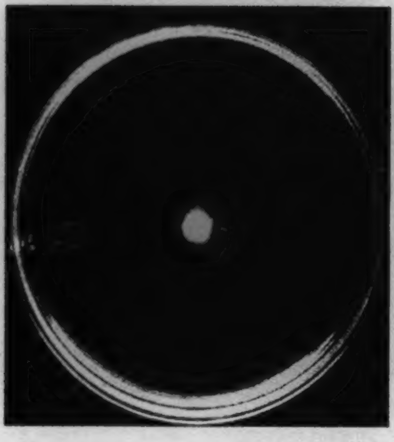
E2348/69 (pBAD18)+glc E2348/69 (pBAD18)+ara

40 minute infection period the bacteria and cells were fixed, permeabilized, and colabelled with anti-Tir(Cy3), anti-EPEC(Alexa 350) antisera, and phalloidin (Alexa 488) a fungal toxin which binds to actin. Figure 3.7 shows an overlay of the three labels from a representative of each slide. The white foci are pedestals, an overlap of EPEC, Tir and actin. Interestingly, arabinose appeared to inhibit EPEC binding to Hela cells as significantly fewer foci were observed in both E2348/69 (pBAD18) and E2348/69 (pROX) grown in arabinose compared to glucose. The same results were observed when the two EPEC strains were grown statically for up to four hours in DMEM+0.2% arabinose in a 5% CO_2 environment, conditions that should induce pedestal formation (data not shown). As few pedestals or bacteria bound to Hela cells were observed when the bacteria were grown in 0.2% arabinose, while numerous pedestals and adhered bacteria are observed during growth in 0.2% glucose, it appears that arabinose inhibits EPEC adherence to Hela cells. Further, when the coverslips were examined under a light microscope prior to fixing, almost no microcolonies were observed when the bacteria were grown in arabinose, while numerous microcolonies were observed when the cells were grown in glucose. Unfortunately, the effect of Cpx activation on pedestal formation could not be assayed as arabinose, required to induce *cpxR* overexpression, appeared to inhibit bacterial binding to the Hela cells.

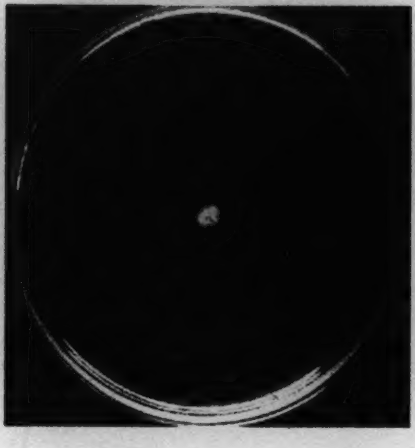
3.3 Swim assay

Previous evidence demonstrated that the Cpx response represses both the *tsr* gene, which codes for a serine chemoreceptor, and an operon containing the motility and chemotaxis genes *motABcheAW* in the *E. coli* strain MC4100 (DeWulf *et al.* 1999). In order to determine whether the Cpx system had a similar effect on motility in EPEC a swim assay was performed on E2348/69 and an E2348/69 *cpxR:kn* mutant. The bacteria were inoculated from a mid-exponential phase culture into a semi solid agar medium which they are able to swim to various degrees through depending on motility, and incubated for 5.5 hours at 37°C. Since inactivation of the *cpxR* gene lead to an increased zone of swimming relative to the isogenic E2348/69 strain, this suggests that the Cpx system repressed the chemotactic and motility genes in EPEC as well as MC4100 (Figure 3.8).

Figure 3.8. Hypermotility of a E2348/69 *cpxR:kn* mutant. E2348/69 and E2348/69 *cpxR:kn* were grown to mid-log phase (OD_{600} 0.3-0.5) shaking in LB at 37°C then 5µl of culture was inoculated into swim agar plates and incubated for 22 hours at 37°C. This experiment was performed twice and a representative result is shown.



E2348/69



E2348/69
cpxR:kn

4. The effect of the Cpx response on EPEC virulence determinant transcription

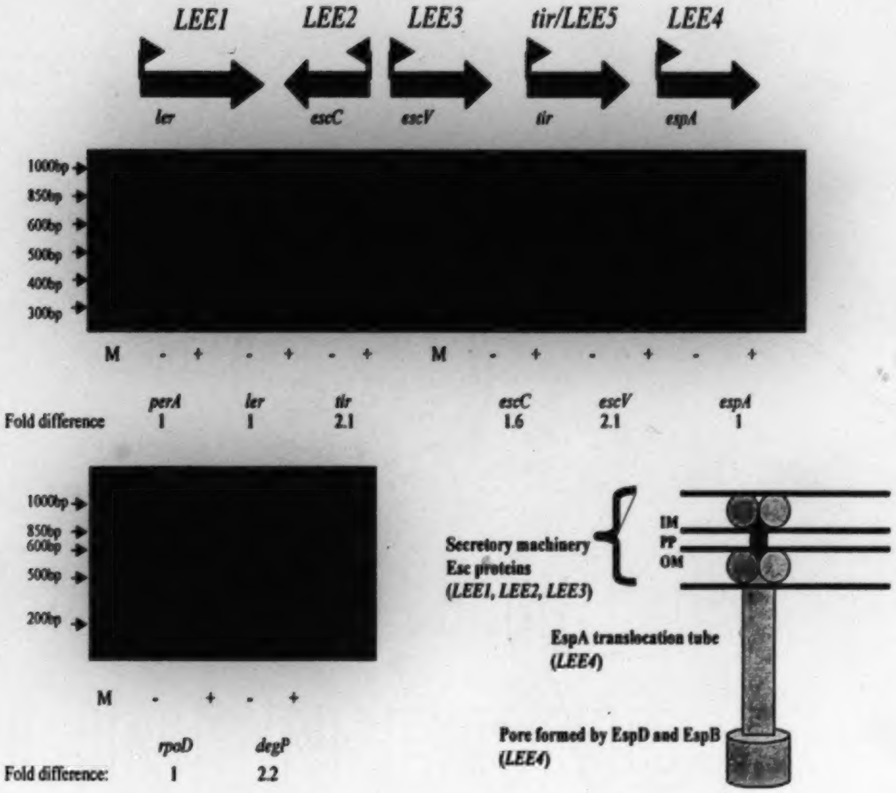
4.1 The effect of the Cpx response on pathogenicity gene transcription

4.1.1. RT-PCR analysis

As Cpx activation in EPEC resulted in increased secretion of EspB and Tir, the transcription of EPEC pathogenicity genes was analyzed to determine whether this reflected increased transcription of either type III secretory apparatus genes, or genes encoding secreted effectors. RT-PCR was carried out on total RNA isolated from an E2348/69 strain with a *cpxR:kn* mutation, carrying either the *cpxR* overexpression vector, pROX, or the vector control, pBAD18, grown at 37°C, shaking at 225 rpm, in LB+ 0.2% arabinose, to an OD_{600} of approximately 0.5. This OD_{600} was chosen as CpxR overexpression from pROX is maximal at this point (Figure 3.1). Initially, numerous controls were performed to prove that the RT-PCR worked as expected. The purity of the RNA was verified by performing an RT-PCR reaction without the reverse transcriptase. RNA concentrations were standardized in all samples according to the level of *rpoD* mRNA, a house keeping sigma factor that is not Cpx-regulated (Fig 4.1). RT-PCR analysis of *degP*, a known member of the CpxR regulon, was performed to confirm that *cpxR* overexpression resulted in a detectable increase in Cpx-regulated gene expression. (Figures 3.1, 4.1). Image densitometry indicated that CpxR overexpression resulted in a 2.2 fold increase in *degP* product abundance (Figure 4.1).

After these controls were completed, RT-PCR analysis using gene-specific primers was carried out to examine expression of a representative member of each of the five LEE operons; *escT, escC, escV, tir, espA* were used for *LEE1*, *LEE2*, *LEE3*,

Figure 4.1. RT-PCR analysis of the affect of CpxR overexpression on the expression of EPEC pathogenicity genes. RNA was isolated from E2348/69 *cpxR:kn* mid-log phase cells containing either the pBAD18 vector control, denoted – , or pROX, a *cpxR* overexpression vector, denoted +, that were grown shaking at 225 rpm in LB+0.2% arabinose at 37°C. To ensure that the concentration of RNA was equal in all samples, an RT-PCR reaction with primers amplifying *rpoD*, coding for σ^{70} of RNA polymerase, was performed and the volumes of the RNA samples were adjusted accordingly. RT-PCR analysis of *degP*, the EPEC regulatory gene *perA*, and a representative gene from the *LEE1 (escT), LEE2* (*escC*), *LEE3* (*escV*), *LEE4* (*espA*) and *tir/LEE5* (*tir*) operons was performed. All RT-PCR reactions consisted of 35 cycles (94°C for 15 s, 55°C for 30 s, and 72°C for 1 min per cycle) with a final extension at 72°C for 10 minutes, and used 0.1µg of RNA as a template. The products were size fractionated on a 1% agarose gel, and bands were visualized through ethidium bromide staining. As a size marker (M), the 1KB+ ladder was used (Invitrogen). The fold difference in band density relative to the vector control is provided, and was determined through image densitometry. The primers used in this experiment are given in Table 2.3. This experiment was repeated two times and the results represent one experiment. A schematic of the LEE operons with the representative gene amplified from each operon is shown. A simplified diagram of the type III secretory apparatus, spanning the bacterial inner membrane (IM), periplasm (P), and outer membrane (OM), is also provided.


LEE1
LEE2
LEE3
tir/LEE5
LEE4
ler
escC
escV
tir
espA
1000bp
850bp
600bp
500bp
400bp
300bp
M - + - + - + M - + - + - +
perA
ler
tir
escC
escV
espA
Fold difference
1
1
2.1
1.6
2.1
1
1000bp
850bp
600bp
500bp
200bp
M - + - +
rpoD
degP
Fold difference:
1
2.2
IM
PP
OM
Secretory machinery
Esc proteins
(LEE1, LEE2, LEE3)
EspA translocation tube
(LEE4)
Pore formed by EspD and EspB
(LEE4)

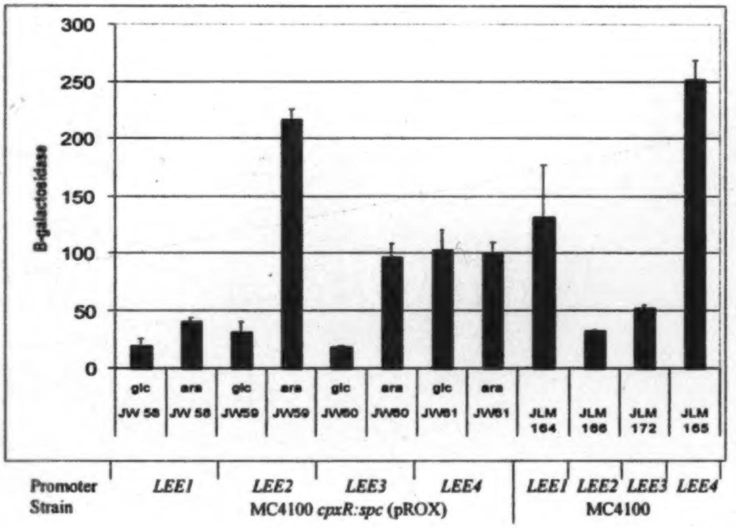
tir/LEE5, and *LEE4*, respectively (Figure 4.1). As transcription of the *LEE1* encoded regulator, Ler, and the bundle-forming pilus operon are positively regulated by the virulence plasmid encoded regulatory locus, *perABC*, RT-PCR analysis was also carried out on the *perA* transcript. The oligonucleotide primers used are listed in Table 2.3. The RT-PCR experiments were performed in duplicate on RNA samples from two independent isolations and a representative result is shown in Figure 4.1. When the *cpxR* response regulator is over-expressed in a *cpxR:kn* mutant, the *tir* and *escV* genes showed a 2.1 fold increase in transcription, while *escC* demonstrated a 1.6 fold increase in transcription by image densitometry. No difference could be detected in *LEE1*, *perA* or *LEE4* transcription. The RT-PCR analysis suggested that during growth in LB, a condition not conducive to virulence gene expression, Cpx-activation causes increased expression of the *LEE2*, *LEE3*, and *LEE5/tir* operons. Since an internal control was not performed in each reaction and quantitative RT-PCR was not used, *lux* and *lacZ* transcriptional fusions were next used to verify the RT-PCR results and to provide a more quantitative analysis of gene expression in response to Cpx activation.

4.1.2 β-galactosidase reporter analysis of LEE operon expression

To date the majority of transcriptional data examining LEE operon expression has been obtained from reporter constructs in the non-pathogenic laboratory strain of *E. coli*, MC4100, allowing for the dissection of LEE regulation in the absence of EPEC-specific regulatory factors. Accordingly, strains containing *LEE1*, *LEE2*, *LEE3*, and *LEE4* fusions to a *lacZ* reporter in the MC4100 chromosome were obtained. The reporters were then moved through P1 transduction onto the chromosome of TR132, an arabinose resistant MC4100 strain carrying an insertionally inactivated *cpxR:spc* allele and pROX, the arabinose-inducible *cpxR* overexpression vector. The expression of the fusions was assayed in the presence and absence of *cpxR* overexpression and compared to the expression observed in a wild-type MC4100 background.

After six hours, *LEE2* showed a 6.8 fold increase in expression when *cpxR* was overexpressed and *LEE3* expression was also increased 5-fold (Figure 4.2). Thus, as observed with the RT-PCR data, CpxR overexpression in the laboratory strain MC4100 also appears to activate expression of the *LEE2* and *LEE3* operons. *LEE5* expression, which increased in response to Cpx activation in the RT-PCR analysis, was not tested. The expression of the *LEE4* fusion was not altered in response to *cpxR* overexpression, however when compared to the activity of the fusion in a wild-type MC4100 background, the activity was repressed 2.5 fold (Figure 4.2). This lowered activity in the MC4100 *cpxR:spc* strain was curious and suggests that CpxR contributes to basal level transcription of the *LEE4* operon in MC4100, even though CpxR overexpression does not appear to increase expression. The activity of the *LEE1* fusion seemed to increase approximately 2 fold in response to *cpxR* overexpression in a *cpxR* null background and, when compared to the MC4100 *LEE1* fusion, the activity of the *LEE1-lux* fusion in a *cpxR:spc* background was 8 fold lower (Figure 4.2). Thus, as with *LEE4* expression in MC4100, CpxR appears to affect basal level transcription of this operon, even though *cpxR* overexpression has a minor effect. However, the diminished LEE1 and LEE4 transcription are not complemented by overexpressing *cpxR*. As the *cpxR* null mutation

Figure 4.2. Examination of LEE gene expression through chromosomal *LEE1, LEE2, LEE3, and LEE4:lacZ* reporter fusions after six hours of growth. Arabinose resistant MC4100 *cpxR:spc* strains transformed with a *cpxR* overexpression plasmid and carrying chromosomal fusions of *LEE1* (JW 58), *LEE2* (JW 59), *LEE3* (JW 60), and *LEE4* (JW61) promoters to *lacZ*, and MC4100 strains carrying the same four chromosomal fusions (JLM 164, JLM 166, JLM 172, and JLM 165, respectively) were diluted 1:50 from an overnight culture grown shaking in LB at 37°C, and grown for six hours in LB shaking at 225 rpm at 37°C. JW 58, JW 59, JW 60, and JW 61 were grown in 0.2% glucose to repress *cpxR* expression from the P_{BAD} promoter and in 0.2% arabinose to induce *cpxR* expression. JLM 164, JLM 166, JLM 172, and JLM 165 were grown in LB without any additional carbon source to obtain the promoter activities for these genes in an MC4100 background. Cells were pelleted by centrifugation at 3220 x g after 6 hours of growth, resuspended in 2ml Z buffer, and lysed by the addition of 100 uL of 1%SDS and 50 uL of chloroform, followed by 30 seconds of vortexing. 50 uL of 10mg/ml ONPG was added and the absorbance at 420 nm was measured every minute for 30 minutes. β-galactosidase levels were determined by dividing the slope of the line obtained by graphing A_{420} vs time by the absorbance of the culture measured at 600 nm. The error bars represent the standard deviation of three independent assays. This experiment was performed once.


300
250
200
150
100
50
0
B-galactosidase
glc
ara
glc
ara
glc
ara
glc
ara
JW 58
JW 58
JW59
JW59
JW60
JW60
JW61
JW61
JLM 164
JLM 166
JLM 172
JLM 165
Promoter
Strain
LEE1
LEE2
LEE3
LEE4
MC4100 cpxR:spc (pROX)
LEE1
LEE2
LEE3
LEE4
MC4100

likely has polar effects on *cpxA*, it is possible that CpxA is required for the Cpx effect on transcription of these operons. Strains carrying the pROX plasmid would only have *cpxR* expression returned, not that of *cpxA*. In an MC4100 *cpxR:spc* background, overexpression of the response regulator, *cpxR*, only increases activity of the *LEE2:* and *LEE3:lacZ* reporter fusions.

4.1.3 Luciferase reporter analysis of LEE operon expression in MC4100

As EPEC virulence determinants are expressed and secreted in a growth phase-dependent manner (Rosenshine *et al.*, 1996), it is important to examine the effect of Cpx activation on virulence gene expression over the course of the growth phase. However RT-PCR is not as conducive to high throughput time courses as reporter fusions are, and *lacZ* fusions pose problems in EPEC as the native *lacZ* gene gives high background in the β-galactosidase assay, obscuring weakly regulated promoters. As well, it is difficult to obtain chromosomal fusions in EPEC, perhaps due to the presence of a restriction system. Therefore, a series of promoter-luciferase fusions were designed with the intent of comparing gene expression in both EPEC and MC4100 backgrounds. The plasmid pNLP10, derived from the low-copy number pCS26 vector (gift from M. Surette) was used, as it contains the full luciferase operon, thus eliminating the need to add substrate as would be required for *luxAB* reporters. pNLP10 differs from pCS26 in that it contains an expanded multiple cloning site, facilitating the creation of reporter vectors. The advantage of the pNLP10-based vectors is that they allow sensitive, real-time, non-invasive examination of gene expression over a time course. The *cpxR*, *perA*, *LEE2*, *LEE3,* and *LEE4* promoters were cloned and inserted into pNLP10 to create the reporter vectors pJW2, pJW6, pJW13, pJW5, and pJW8, respectively.

To ensure that the pNLP10-based reporters were giving accurate information about LEE gene expression, relative levels of gene expression were measured in TR132, the arabinose resistant MC4100 *cpxR:spc* strain carrying the *cpxR* overexpression plasmid, and the trends in gene expression were compared to those seen with the *lacZ* reporters (section 4.1.2). Overnight cultures of the TR132 transformants carrying the newly constructed *lux* reporter fusions were diluted 1:50 into L-broth containing either 0.2% glucose or 0.2% arabinose and grown shaking at 225 rpm at 37°C. Luminescence and OD_{600} were taken after 6 hours of growth, and the data was normalized by dividing luminescence by OD_{600}. The Cpx-regulated *cpxR* promoter exhibited between 5 and 8 fold induction. This is higher than the 2-4 fold induction observed in the literature examining *cpxR:lacZ* reporter fusion activity in response to Cpx activation (Otto and Silhavy, 2002; DiGiuseppe and Silhavy, 2003). The inductions of the *LEE2, LEE3,* and *perA* reporters are 445-643 fold, 36-84 fold, and 12-14 fold in response to *cpxR* overexpression, respectively. Not only are these induction levels higher than those observed for *cpxR*, but they are also higher than the 15-fold induction reported for a *cpxP-lacZ* reporter in response to Cpx activation (DiGiuseppe and Silhavy, 2003). This is especially noteworthy as *cpxP* is the most inducible member of the Cpx regulon identified to date. The *lacZ* fusions of *degP, dsbA, spy,* and *ppiA* all have similar levels of induction, between 2-4 fold, in response to Cpx activation (DiGiuseppe and Silhavy, 2003). It should be noted, however, that these induction values are in response to elevated pH and adhesion, known Cpx-activating signals, and the induction ratios for these genes in response to CpxR overexpression have not been compared. Thus, one possible explanation for the large induction ratios seen with the *lux* reporters may be that CpxR overexpression is a far more potent mechanism of activating the Cpx response. Another possible explanation for the large induction ratios observed in these experiments relative to those performed previously on known Cpx-regulated genes is that the *lux* reporters may be much more sensitive than the *lacZ* reporters. The *LEE2* and *LEE3 lacZ* reporters had 6.8 fold and 5 fold induction, respectively, while the equivalent *lux* reporters had 445-643 fold, 36-84 fold inductions respectively.

The *LEE4* reporter showed no induction in response to *cpxR* overexpression (Table 4.1). The product of the *perABC* locus activates expression of the LEE encoded positive regulator Ler, which in turn activates expression of *LEE2, LEE3,* and *LEE4*. The 12-14 fold induction of the *perA* reporter is important as a change in PerA levels would have huge downstream effects on virulence gene expression through the Ler regulator. Similar to the *lacZ* reporter data, the *lux* reporters demonstrated that *LEE2* and *LEE3* reporter activity increases in response to Cpx activation, as did the activity of the *perA:lux* fusion, a promoter not examined through *lacZ* reporter activity.

4.1.4 Luciferase reporter analysis of LEE operon expression in EPEC

Murphy and Campellone (2003) recently observed that they were unable to transform EPEC with either of two pSC101-origin containing plasmids. Similarly, all attempts to introduce the pSC101origin-containing, pNLP10-based *lux* reporter plasmids described in the previous section into the EPEC wild-type strain E2348/69 failed. As the origin appeared to be either incompatible with the resident plasmids of EPEC or unstable

Table 4.1. Effect of Cpx activation on EPEC pathogenicity gene expression in MC4100.

Experiment[1]	Promoter[2]	*cpxR* overexpression	CPS/OD600[3]	Std Dev[4]	Fold difference in response to *cpxR* overexpression
1	pNLP10	-	714	365.45	
1	pNLP10	+	187	114.22	0.26
2	pNLP10	-	420	226.78	
2	pNLP10	+	90	57.63	0.21
1	*CpxR*	-	3471	324.06	
1	*CpxR*	+	18006	682.38	5.19
2	*CpxR*	-	2457	478.66	
2	*CpxR*	+	20144	1542.19	8.20
1	*Per*	-	890	362.25	
1	*Per*	+	10671	661.23	11.98
2	*Per*	-	1369	120.44	
2	*Per*	+	19178	1079.99	14.01
1	*LEE2*	-	302	273.75	
1	*LEE2*	+	134240	3204.73	445.13
2	*LEE2*	-	209	246.71	
2	*LEE2*	+	134394	2773.98	643.41
1	*LEE3*	-	809	76.63	
1	*LEE3*	+	29496	1841.35	36.46
2	*LEE3*	-	373	209.69	
2	*LEE3*	+	31398	2232.71	84.22
1	*LEE4*	-	3722	878.94	
1	*LEE4*	+	7053	249.01	1.90
2	*LEE4*	-	972	127.06	
2	*LEE4*	+	2120	280.66	2.18

1) The results from two experiments are shown.

2) MC4100 *cpxR:spc*(pROX) carrying the indicated *lux* transcriptional fusions, and the vector control, pNLP10,were grown in LB containing 0.2% glucose (-) or arabinose (+) to repress or induce *cpxR* overexpression, respectively.

3) After 6 hours of growth at 37°C, shaking at 225 rpm, the luminescence was read (CPS) and normalized using the OD_{600}. Each value is the average of five samples taken from a culture inoculated with five independent colonies.

4) Standard deviation was calculated from the five separate samples used to derive the average CPS/OD_{600}.

in EPEC, the pSC101 origin was removed from pNLP10 through digestion with *Pac*I. The p15A origin was amplified from pACYC184 using *PacI* tagged primers and cloned in place of the pSC101 origin on pNLP10 to create pJW15. This origin was chosen as it is from a low copy number vector (approximately 15), and has been demonstrated in the literature to be compatible in EPEC (Sambrook *et al.*, 1989; Gómez-Duarte and Kaper; 1995; Grant *et al.*, 2003). As expected, transformation of pJW15 into EPEC resulted in numerous transformants. Thus, the same *LEE2, LEE3, LEE4, perA*, *bfpA,* and *cpxR* promoter fragments used to construct the pNLP10-based reporter plasmids (section 4.1.3) were cloned into pJW15 to create pJW18, pJW19, pJW20, pJW22, pJW23 and pJW24, respectively. These plasmids were electroporated into E2348/69 which had been heat treated for 30 minutes at 50°C during preparation of electrocompetent cells in order to temporarily inactivate any restriction systems that might be present. Once in EPEC, and thus modified by any host methylation systems that might exist, the plasmids were isolated and transformed into E2348/69, JPN15, and E2348/69Δ*ler* that carried the vector control pBAD18, the *nlpE* overexpression plasmid pND18, or the *cpxR* overexpression vector pROX. JPN15 lacks the EAF plasmid that encodes the *bfp* and *perABC* operons, and E2348/69Δ*ler* contains a deletion of the *ler* gene.

Since the p15 origin has a higher copy number than the pSC101 origin, an experiment was performed to analyze the effect of copy number on reporter activity. The *LEE1* promoter region from pJLM164 (Mellies *et al.*, 1999) was cloned into both pNLP10 and pJW15 and these constructs were electroporated into MC4100 *cpxR:spc* (pROX). The expression of the *LEE1:lux* fusion from the pJW15-based fusion was compared to the expression of the *LEE1:lux* fusion encoded on the pNLP10-based vector

Table 4.2. Effect of copy number on *lux* reporter activity.

Plasmid[1]	*cpxR* overexpression	CPS/OD600[2]	Std Dev[3]	Fold difference in response to *cpxR* overexpression[4]
pNLP10	-	377	79.59	
pNLP10	+	1119	581.11	2.97
pJW15	-	5886	757.73	
pJW15	+	6570	955.89	1.12
pJW3	-	524	138.53	
pJW3	+	13442	1188.41	25.66
pJW17	-	19102	7609.15	
pJW17	+	133144	2725.01	6.97

1) MC4100 *cpxR:spc*(pROX) carrying the the vector control, pNLP10, the *LEE1:lux* fusion in pNLP10 (pJW3), the vector control, pJW15, and the *LEE1:lux* fusion in pJW15 (pJW17), were grown in LB containing 0.2% glucose (-) or arabinose (+) to repress or induce *cpxR* overexpression, respectively.

2) After 6 hours of growth at 37°C, shaking at 225 rpm, the luminescence was read (CPS) and normalized using the OD_{600}. Each value is the average of five samples taken from the same culture that was inoculated from five independent colonies.

3) Standard deviation was calculated from the five replicates that were used to obtain the average CPS/OD_{600}.

4) The experiment was performed once.

in the same strain background to ensure that copy number did not alter the accuracy of luminescence. After 8 hours of growth at 37ºC, shaking at 225rpm, in the presence of 0.2% arabinose to induce *cpxR* overexpression, both *LEE1* reporters showed induction in response to *cpxR* overexpression (Table 4.2). LEE1 expression was induced approximately 25 fold from the pNLP10-based vector and approximately 7 fold from the pJW15-based plasmid (Table 4.2). Thus, the higher copy number of pJW15 appears to decrease sensitivity (Table 4.2). Copy number had a major effect on luminescence as the *LEE1:lux* reporter in pJW15 had a 10-20 fold increase in luminescence relative to the same reporter in pNLP10. Interestingly a large increase in *LEE1* promoter activity was observed in MC4100 using the *lux* reporters that was not detected using *lacZ* reporters in MC4100 or RT-PCR in EPEC. As EPEC contains numerous regulatory factors absent in MC4100, no Cpx effect may have been observed in RT-PCR analysis in EPEC due to the overwhelming effect of other positive regulators, such as Per. The *lux* reporters appear to be much more sensitive than the *lacZ* reporters, explaining why a Cpx effect on *LEE1* promoter activity was only seen using *lux* reporters, not *lacZ* reporters. The pJW15 based reporters, although less sensitive than the lower copy pNLP10 reporters, which are not compatible with EPEC, are still more sensitive than *lacZ* reporters.

Since the pJW15 reporters appeared to accurately reflect Cpx-mediated increases in expression, LEE-*lux* reporter expression was next analyzed in an EPEC background to determine if the Cpx pathway might affect LEE gene expression. All luciferase assays in EPEC were initially conducted as timecourses, with readings taken every hour after subculturing into fresh media. All Cpx-dependent effects were observed during mid-exponential phase, between 2 and 4.5 hours of growth, with no significant changes observed earlier and loss of the Cpx effect at later growth points. This is in agreement with previous immunoblotting and RT-PCR experiments (Fig 3.1) which demonstrated a similar time frame for activation of the Cpx pathway by *cpxR* or *nlpE* overexpression. Cpx-dependent reporter expression in the MC4100 *cpxR:spc* strain was observed after six hours of growth, while the Cpx-effect in EPEC strains occurred between 2 and 4.5 hours. There are many differences between the EPEC and MC4100 strains that might account for this apparent discrepancy. MC4100 lacks the positive virulence regulators Per and Ler, and the experiments in the MC4100 background were done in a *cpxR* mutant while the EPEC background was wild-type with respect to the Cpx pathway. As well, the Cpx pathway in EPEC has not been characterized, so it is not known if the EPEC Cpx pathway functions in an identical manner to the MC4100 pathway. Since the Cpx-dependent effect on virulence gene expression was observed between the 2 and 4.5 hour timepoints, corresponding to an OD_{600} of between 0.2 and 0.5, these are the timepoints shown in this section.

The *cpxR:lux* fusion in pJW15 had only a 1.5-1.6 fold increase in response to *cpxR* overexpression in EPEC (Table 4.3). This is contrasted with the 5-8 fold increase the *cpxR:lux* fusion carried on pNLP10 exhibited in response to *cpxR* overexpression in MC4100. Copy number of pJW15 may play a role in this diminished induction, as the pJW15 reporters are less sensitive than the pNLP10 reporters. As well, the nature of the Cpx response in EPEC has not been well characterized. The *cpxR* promoter shows the weakest level of induction in response to Cpx pathway activation of any Cpx-regulated gene characterized to date (Raivio et al. 1999) and could be even more weakly regulated

Table 4.3. Effect of Cpx activation on pathogenicity gene expression in EPEC.

Exp. Number[1]	Media	Promoter[2]	Plasmid	CPS/OD_{600}[3] (x 10^5)	Std Dev[4] (x 10^4)	Fold difference: relative to pBAD18
1	DMEM	*bfpA*	pBAD18	5.43	9.66	
1	DMEM	*bfpA*	pND18	13.0	15.1	2.39
1	DMEM	*bfpA*	pROX	12.6	7.83	2.33
1	LB	*bfpA*	pBAD18	1.42	0.493	
1	LB	*bfpA*	pND18	1.41	0.844	0.99
1	LB	*bfpA*	pROX	2.00	7.30	1.41
2	DMEM	*bfpA*	pBAD18	5.24	2.93	
2	DMEM	*bfpA*	pND18	8.00	2.88	1.53
2	DMEM	*bfpA*	pROX	12.6	4.59	1.58
2	LB	*bfpA*	pBAD18	1.98	3.15	
2	LB	*bfpA*	pND18	1.58	0.531	0.80
2	LB	*bfpA*	pROX	3.96	5.41	2.00
1	DMEM	*LEE2*	pBAD18	8.50	9.37	
1	DMEM	*LEE2*	pND18	12.9	25.1	1.52
1	DMEM	*LEE2*	pROX	15.4	12.5	1.82
1	LB	*LEE2*	pBAD18	1.76	0.398	
1	LB	*LEE2*	pND18	1.06	1.00	0.60
1	LB	*LEE2*	pROX	1.37	0.757	0.78
2	DMEM	*LEE2*	pBAD18	7.55	10.9	
2	DMEM	*LEE2*	pND18	11.2	16.3	1.49
2	DMEM	*LEE2*	pROX	17.4	25.2	2.31
2	LB	*LEE2*	pBAD18	1.58	1.49	
2	LB	*LEE2*	pND18	0.944	0.366	0.60
2	LB	*LEE2*	pROX	0.746	0.187	0.47
1	DMEM	*LEE3*	pBAD18	6.16	1.44	
1	DMEM	*LEE3*	pND18	18.6	7.34	3.02
1	DMEM	*LEE3*	pROX	17.3	34.5	2.80

Exp. Number[1]	Media	Promoter[2]	Plasmid	CPS/OD600[3] (x 10^5)	Std Dev[4] (x 10^4)	Fold difference: relative to pBAD18
1	**LB**	*LEE3*	pBAD18	0.680	0.549	
1	LB	*LEE3*	pND18	0.286	0.384	0.42
1	LB	*LEE3*	pROX	1.12	2.08	1.65
2	DMEM	*LEE3*	pBAD18	8.21	3.79	
2	DMEM	*LEE3*	pND18	22.4	6.03	2.74
2	DMEM	*LEE3*	pROX	25.8	8.31	3.14
2	LB	*LEE3*	pBAD18	1.44	0.540	
2	LB	*LEE3*	pND18	0.768	0.155	0.53
2	LB	*LEE3*	pROX	2.62	2.08	1.83
1	DMEM	*LEE4*	pBAD18	2.02	2.37	
1	DMEM	*LEE4*	pND18	8.01	2.01	3.96
1	DMEM	*LEE4*	pROX	3.48	1.31	1.72
1	LB	*LEE4*	pBAD18	1.97	0.298	
1	LB	*LEE4*	pND18	1.45	0.569	0.73
1	LB	*LEE4*	PROX	2.30	1.36	1.17
2	DMEM	*LEE4*	pBAD18	2.52	60.6	
2	DMEM	*LEE4*	pND18	8.00	2.88	3.17
2	DMEM	*LEE4*	pROX	4.09	1.07	1.62
2	LB	*LEE4*	pBAD18	1.80	0.289	
2	LB	*LEE4*	pND18	1.58	0.531	0.88
2	LB	*LEE4*	pROX	1.66	0.290	0.92
1	DMEM	*cpxR*	pBAD18	3.27	0.953	
1	DMEM	*cpxR*	pROX	4.88	6.02	1.49
1	LB	*cpxR*	pBAD18	1.12	0.273	
1	LB	*cpxR*	pROX	1.79	1.24	1.60

1) The results from two experiments are shown.
2) E2348/69 strains carrying the vector control, pBAD18, the *nlpE* overexpression vector, pND18, and the *cpxR* overexpression vector, pROX, as well as the indicated *lux* transcriptional fusions, were grown in DMEM or LB containing 0.2% arabinose and grown shaking at 225 rpm, at 37°C, until an OD_{600} of 0.2-0.5 was reached.
3) The luminescence was read (CPS) and normalized using the OD_{600}. Each value is the average of five readings taken from five samples of a culture grown from five independent colonies.
4) Standard deviation was calculated from the CPS/OD_{600} values used to obtain the average CPS/OD_{600}.

by the Cpx response in EPEC. As this fusion was only analyzed once, it must be repeated before any inferences are made. A *cpxP* fusion, the Cpx regulon member with the largest induction in response to Cpx activation (DiGiuseppe et al., 2003), is being constructed in pJW15 to provide a better positive control. The *cpxR:lux* fusion suggests that the Cpx response is very weakly activated in EPEC in response to *cpxR* overexpression, though this control is not conclusive as the induction is lower than expected. In EPEC, the *LEE1:lux* fusion had a 10 fold higher activity than the *LEE2:lux* fusion, which does not agree with the recent literature which showed that a *LEE2:gfp* fusion had a 1.7 fold higher activity than a *LEE1:gfp* fusion (Umanski *et al.*, 2002) (data not shown). A possible reason for this difference in expression is that the *lux* fusion was constructed using a 726 basepair fragment of the *LEE1* promoter, from -505 to +221, while the *gfp* fusion contained a 2092 basepair promoter fragment, from –1673 to +419. The extra upstream and downstream sequence could contain regulatory regions missing in the *lux* fusion, accounting for the differential expression of the fusions. As the *LEE1:lux* fusion did not behave as expected, it was only used to analyze the effect of copy number on reporter activity. A new *LEE1:lux* fusion is currently being constructed.

As expected, the *bfpA* promoter, which was inactive in MC4100 (data not shown) was highly active in EPEC (Table 4.3). The *bfpA:lux* fusion produced the highest levels of activity observed for any of the reporters, with CPS/OD_{600} values in the millions (Table 4.3). There was a 1.5 to 2.4 fold increase in *bfpA* reporter activity when *cpxR* or *nlpE* were overexpressed relative to the vector control and the bacteria were grown in DMEM. Growth in LB produced only a 3 fold lower level of activity compared to growth in DMEM (Table 4.3). When *nlpE* or *cpxR* were overexpressed during growth in

LB, little to no change was observed in *bfpA:lux* activity (Table 4.3). Thus, the *bfp* operon appears to be minimally affected by activation of the Cpx response or overexpression of *cpxR*.

When the bacteria were grown in DMEM, the *LEE2* fusion showed between a 1.8 and 2.3 fold increase in activity when *cpxR* was overexpressed and a 1.5 fold increase when *nlpE* was overexpressed, with respect to the same strain carrying the vector control (Table 4.3). The activities of the *LEE2:lux* fusion in E2348/69 strains overexpressing *cpxR* during LB growth were between 1.3 and 2.1 fold depressed in expression, while *nlpE* overexpression in LB caused a 1.6 fold decrease in expression (Table 4.3). The expression of the *LEE3* operon had between a 2.8 and 3.1 fold increase in expression in the presence of *cpxR* and *nlpE* overexpression with respect to the vector control (Table 4.3) when the bacteria were grown in DMEM. Only a 1.6-1.8 fold increase in activity was observed when growth took place in LB in response to *cpxR* overexpression (Table 4.3). When grown in LB, the *LEE3* reporter appeared to have a 1.4-1.9 fold decrease in activity in response to *nlpE* overexpression (Table 4.3). This effect is contrasted with the 445-643 fold and 36-84 fold induction observed in response to *cpxR* overexpression in MC4100 *cpxR:spc* using *LEE2:lux* and *LEE3:lux* fusions, respectively, in pNLP10. As well, the *LEE2* and *LEE3:lacZ* fusions had 6.8 and 5 fold increases, respectively, in response to *cpxR* overexpression in MC4100 *cpxR:spc*. The large increase in *LEE2* and *LEE3* expression observed in MC4100 in response to Cpx activation does not occur in EPEC. The absence of EPEC specific regulators, such as Ler and Per, in MC4100 may allow for a Cpx effect that is not seen in EPEC due to the presence of these regulators and the consequent increase in expression of *LEE2* and *LEE3* (i.e. the background level of gene expression is much higher). The RT-PCR data, demonstrating a 1.6 and 2.1 fold increase in *LEE2* and *LEE3* expression in response to *cpxR* overexpression in E2348/69 *cpxR:kn,* suggests a minor Cpx effect on these two operons in EPEC, not the degree implied by the *lux* and *lacZ* reporters in MC4100. *LEE2* and *LEE3* expression appears to be affected by Cpx activation on a minor level in EPEC.

LEE4, which was not affected by CpxR overexpression in MC4100 or EPEC by RT-PCR analysis, had a 1.6-1.7 fold increase in reporter activity in E2348/69 strains overexpressing *cpxR* that were grown in DMEM compared to the vector control (Table 4.3). No change was observed in *LEE4:lux* expression between EPEC strains carrying the vector control, pBAD18, the *nlpE* overexpression vector, pND18 and the *cpxR* overexpression vector, pROX, in LB. Interestingly when *nlpE* was overexpressed during growth in DMEM the *LEE4:lux* fusion exhibited a 3.1-4 fold induction (Table 4.3). Thus, Cpx activation through *cpxR* overexpression has no effect on *LEE4* expression in EPEC, while *nlpE* overexpression leads to increased expression of the *LEE4:lux* fusion.

The use of *lacZ* and *lux* reporters in MC4100 had suggested that the Cpx response had a major effect on the expression of the *LEE2* and *LEE3* operons. When the activity of pathogenicity gene reporter fusions were examined in EPEC the results were not suggestive of a strong transcriptional Cpx effect. The positive control, *cpxR* had a very low level of expression, much lower than that seen in MC4100. Without a better positive control, such as *cpxP,* it is difficult to make conclusions about the significance of a 2-fold or less induction in response to Cpx activation in EPEC. pJW15 reporters are less sensitive than pNLP10 reporters, which may partially explain the lower induction levels.

The *lux* reporters in EPEC do not provide convincing evidence of a Cpx effect on transcription of pathogenicity genes.

4.1.5 Reporter analysis of virulence gene regulation in EPEC

The *perABC* locus is found on the EAF plasmid and encodes a regulator, Per, which activates the expression of the *LEE1* operon that contains the gene encoding the positive regulator, Ler (Figure 4.3). Ler alleviates H-NS repression of the remaining LEE operons (Figure 4.3). Since the expression of LEE2 and LEE3 appeared to be slightly increased by Cpx activation in EPEC, the expression of the *perA* promoter was examined to determine whether the Cpx effect was Per-mediated. *perA* promoter activity exhibited a 1.6-2.1 fold induction when CpxR was overexpressed during DMEM growth and a 2.1-2.5 fold induction in LB when *cpxR* was overexpressed (Table 4.4). When *nlpE* was overexpressed there was a 1.4 fold induction when grown in DMEM and no change when grown in LB (Table 4.4). Like the *LEE2* and *LEE3* reporters, the *perA* reporter exhibited only a minor induction in response to *cpxR* overexpression in EPEC while a larger change, 11-14 fold, was observed in MC4100. The inductions for *perA*, *LEE2*, and *LEE3* are lower than the 2-4 fold inductions observed for *lacZ* reporters of *cpxR*, *degP*, *dsbA*, *spy*, and *ppiA* (DiGiuseppe and Silhavy, 2003). Without *lux* reporter-fusions for more Cpx regulated genes in EPEC it is not possible to conclude that the Cpx pathway has an effect on *perA* transcription in EPEC. Given that the *lux* reporters exhibit increased sensitivity to inducing cues, relative to the *lac* reporters, it seems unlikely that these induction values are significant.

Ler is also centrally involved in virulence gene regulation in EPEC (Figure 4.3). To further examine whether the Cpx response might affect *perABC* expression, and to test whether the Cpx pathway might influence transcription of virulence genes via Ler, the activity of the *LEE3, bfpA,* and *perA* fusions were examined in EPEC strains which lacked either the *perABC* containing EAF plasmid, JPN15, or the Ler regulator, E2348/69Δ*ler*. In addition, each of these strains carried either the *cpxR* overexpression plasmid, pROX, or the vector control, pBAD18. The *LEE3* reporter was chosen as it demonstrated the strongest induction in response to *cpxR* overexpression in EPEC and its expression is controlled by Ler, thus *LEE3:lux* activity would be indicative of Ler levels in the cell. The expression of *bfpA* is solely regulated by *perA,* thus the activity of this reporter should mirror the expression of the *perA:lux* fusion, which was included as *perA* encodes the major activator of *ler* expression. The presence of the *perABC* operon seemed to have an effect on *LEE3* expression, since, in contrast to EPEC, in which *cpxR* overexpression from pROX lead to a 2.8-3.1-fold increase in LEE3 expression, Cpx activation in JPN15, the strain lacking the *bfp/per* encoding EAF plasmid, had no effect on the activity of the *LEE3:lux* reporter (Table 4.4). The absence of PerABC severely diminished *bfpA* expression, reducing it 13-82 fold and *perA* expression was reduced 1.7-4.5 fold, as expected, since these genes have previously been shown to be Per-regulated (Martinez-Laguna *et al.*, 1999). Again, overexpression of *cpxR* had no effect on these reporters in a JPN15 background as the *perA* reporter had a 0.84-1.09 fold change in expression, while the *bfpA* reporter had a 0.81 fold decrease in activity. In a wild-type background *cpxR* overexpression results in a 2.1 fold and a 1.6-2.3 fold increase in *perA* and *bfpA* reporter activity, respectively. This suggests that the minor Cpx effect seen on

Figure 4.3. Roles of Per and Ler in the EPEC regulatory cascade. The EAF plasmid encoded regulator, Per, is a transcriptional activator of *LEE1*. The *LEE1* encoded regulator, Ler, then activates transcription of *LEE2, LEE3, LEE4,* and *tir/LEE5.*

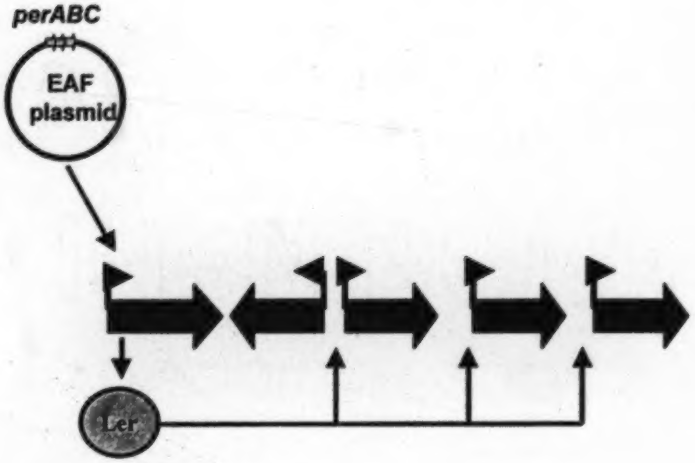

perABC
EAF plasmid
Ler

Table 4.4: The role of the EPEC regulators Per and Ler in the Cpx effect on transcription of EPEC pathogenicity genes.

Exp. Number[1]	Media	Promoter[2]	Plasmid	CPS/OD$_{600}$[3] (x 10^5)	Std Dev[4] (x 10^4)	Fold difference: relative to pBAD18
1	DMEM	*perA*	E2348/69(pBAD18)	4.22	3.53	
1	DMEM	*perA*	E2348/69(pND18)	5.77	3.92	1.37
1	DMEM	*perA*	E2348/69(pROX)	8.94	13.8	2.12
1	LB	*perA*	E2348/69(pBAD18)	1.30	1.00	
1	LB	*perA*	E2348/69(pND18)	1.38	0.351	1.06
1	LB	*perA*	E2348/69(pROX)	2.76	1.15	2.13
2	DMEM	*perA*	E2348/69(pBAD18)	4.52	1.62	
2	DMEM	*perA*	E2348/69(pND18)	6.48	4.98	1.43
2	DMEM	*perA*	E2348/69(pROX)	7.24	6.79	1.60
2	LB	*perA*	E2348/69(pBAD18)	1.35	0.566	
2	LB	*perA*	E2348/69(pND18)	1.25	0.391	0.92
2	LB	*perA*	E2348/69(pROX)	3.33	0.789	2.46
1	DMEM	*perA*	JPN15 (pBAD18)	2.36	0.379	
1	DMEM	*perA*	JPN15 (pROX)	1.98	1.40	0.84
1	DMEM	*perA*	E2348/69 Δ*ler* (pBAD18)	32.3	14.6	
1	DMEM	*perA*	E2348/69 Δ*ler* (pROX)	33.7	10.4	1.17
2	DMEM	*perA*	JPN15 (pBAD18)	2.70	1.91	
2	DMEM	*perA*	JPN15 (pROX)	2.96	4.14	1.09
2	DMEM	*perA*	E2348/69 Δ*ler* (pBAD18)	19.5	11.2	
2	DMEM	*perA*	E2348/69 Δ*ler* (pROX)	23.8	15.4	1.22

Exp. Number[1]	Media	Promoter[2]	Plasmid	CPS/OD$_{600}$[3] (x 10^5)	Std Dev[4] (x 10^4)	Fold difference: relative to pBAD18
1	DMEM	*bfpA*	JPN15 (pBAD18)	0.190	0.147	
1	DMEM	*bfpA*	JPN15 (pROX)	0.153	0.152	0.81
1	DMEM	*bfpA*	E2348/69 Δ*ler* (pBAD18)	50.3	4.04	
1	DMEM	*bfpA*	E2348/69 Δ*ler* (pROX)	49.5	18.5	0.98
2	DMEM	*bfpA*	JPN15 (pBAD18)	0.409	0.147	
2	DMEM	*bfpA*	JPN15 (pROX)	0.329	0.152	0.81
2	DMEM	*bfpA*	E2348/69 Δ*ler* (pBAD18)	33.1	4.04	
2	DMEM	*bfpA*	E2348/69 Δ*ler* (pROX)	41.2	18.5	1.24
1	DMEM	LEE3	JPN15 (pBAD18)	5.64	4.27	
1	DMEM	LEE3	JPN15 (pROX)	6.18	6.42	1.10
1	DMEM	LEE3	E2348/69 Δ*ler* (pBAD18)	2.18	1.06	
1	DMEM	LEE3	E2348/69 Δ*ler* (pROX)	3.33	3.42	1.53
2	DMEM	LEE3	JPN15 (pBAD18)	9.13	2.81	
2	DMEM	LEE3	JPN15 (pROX)	8.47	2.58	0.93
2	DMEM	LEE3	E2348/69 Δ*ler* (pBAD18)	3.42	1.30	
2	DMEM	LEE3	E2348/69 Δ*ler* (pROX)	5.89	2.26	1.72

1) The results from two experiments are shown.
2) E2348/69 strains carrying the vector control, pBAD18, the *nlpE* overexpression vector, pND18, and the *cpxR* overexpression vector, pROX, as well as the *perA:lux* transcriptional fusions, were grown in DMEM or LB containing 0.2% arabinose and grown shaking at 225 rpm, at 37°C, until an OD$_{600}$ of 0.2-0.5 was reached. EPEC strains JPN15, which lacks the *perABC* encoding EAF plasmid, and E2348/69Δ*ler*, carrying pBAD18 and pROX, as well as the indicated *lux* transcriptional fusion, were grown in the same manner.
3) The luminescence was read (CPS) and normalized using the OD$_{600}$. Each value represent the average of five measurements taken from a single culture grown from five independent colonies.
4) Standard deviation was calculated from these five replicates.

bfpA and *perA* activity requires PerABC as in its absence no induction is observed.

The deletion of *ler* reduced LEE3 reporter activity to near MC4100 levels and only a very minor activation could be seen in the *cpxR* overexpressing strain. When *cpxR* was overexpressed in the *ler* deletion strain, a 1.53-1.72 fold increase in LEE3 expression was seen relative to the vector control, as compared to a 2.8-3.1 fold increase seen in the EPEC background (Table 4.4). As well, the *LEE3* reporter had a 4.4-5 fold lower activity in the *ler* deletion strain relative to wildtype EPEC (Table 4.3, Table 4.4). Thus, Ler is required for both maximal *LEE3* activity, and the Cpx effect on *LEE3* expression.

Interestingly the deletion of *ler* caused a massive increase in both *bfpA* and *perA* expression (Table 4.4). Though the minor Cpx effect observed in E2348/69 was not discernable in the Δ*ler* background there was a 5-10 fold increase in expression of both fusions relative to a wild-type EPEC background (Table 4.3, Table 4.4). This suggests that Ler has a negative effect on expression of *perA*, the decreased levels of which likely result in lower expression of *bfpA*. Alternatively, Ler may directly affect the *bfpA* promoter itself.

Cumulatively, the data from the strains lacking *perABC* and *ler* suggest that the Cpx effect on *LEE3* expression requires the presence of both the Per and Ler regulators. In the absence of Per, the expression of *bfpA* and *perA* is severely attenuated and no Cpx-dependent change in expression is evident, while in the absence of Ler there is a large increase in expression of both fusions, though again, no Cpx-dependent increase in expression is evident.

In this chapter, the effect of Cpx activation on EPEC pathogenicity gene transcription was analyzed using both RT-PCR and *lux* reporters. The expression of the *perA* gene, and *LEE2, LEE3,* and *LEE4* operons was examined using both techniques. RT-PCR analysis showed no increase in expression of *perA,* or *LEE4* in response to *cpxR* overexpression when the bacteria were grown in LB while *LEE2* and *LEE3* operon product abundance increased 1.6 and 2.1 fold, respectively (Figure 4.2). When grown in LB, the *LEE4:lux* reporter did not increase in expression in response to Cpx activation, consistent with the RT-PCR data, however the *perA:lux* reporter expression increased 2.1-2.5 fold in response to Cpx activation (Table 4.3, Table 4.4). A possible explanation is that increased promoter activity in response to Cpx activation does not lead to an increase in *perA* transcript detectable through RT-PCR. As the level of *perA* induction in response to Cpx activation in EPEC does not approach the level seen in MC4100, the Cpx effect on secretion may not be mediated through activation of *perA*. The *LEE2:lux* reporter exhibited decreased activity in response to *nlpE* or *cpxR* overexpression when grown in LB, relative to the vector control, and the *LEE3:lux* reporter increased 1.7 fold when *cpxR* was overexpressed in LB. These changes in expression in response to CpxR or NlpE overexpression are markedly less than the 1.6- and 2.1-fold increase in LEE2 and LEE3 expression observed in the RT-PCR analysis in response to CpxR overexpression (Table 4.3, Figure 4.1). Although the *lux* reporter and RT-PCR data display some differences, it must be noted that the RT-PCR was performed in a *cpxR* null strain of EPEC, while the *lux* assays were conducted in wild-type EPEC. A Cpx-dependent effect on transcription would be more detectable in a *cpxR* null strain because of lowered background expression of Cpx-regulated genes in the absence of the response regulator.

The higher copy number of the pJW15-based *lux* reporters renders them less sensitive as well, so any differences in transcription during growth in LB, a condition not conducive to virulence, may be difficult to detect. When the *lux* reporters were examined in DMEM, which is conducive to virulence gene expression, similar increases in expression of the LEE2 and LEE3 promoters are seen relative to those observed in the RT-PCR (Table 4.3).

Analysis of the *lux* reporters in MC4100 suggested large Cpx-dependent increases in expression in the *LEE2*, *LEE3*, and *perA* promoters while the use of *lux* reporters in EPEC failed to detect major differences in expression of these promoters. The fold changes observed in these promoters ranged from 11-14 fold for *perA* to 445-643 fold for *LEE2*. In EPEC the largest induction observed was approximately 3 fold, for *LEE3*. As well, though the reporters tended to have a 100 fold higher luminescence in EPEC compared to MC4100, it would be worthwhile to perform the MC4100 reporter experiments with pJW15-based reporters so as to separate copy number effects from differences between MC4100 and EPEC expression. The *LEE4* promoter fusion showed no change in expression in response to *cpxR* overexpression in either EPEC or MC4100. The Cpx effect observed in MC4100 in response to *cpxR* overexpression was not observed using *lux* reporters in EPEC.

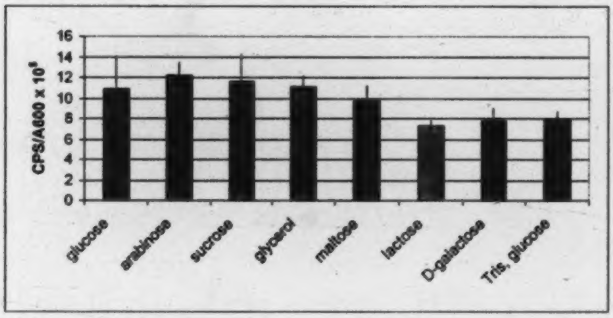
4.2 The effect of carbon source on *perA* and *bfpA* expression

As growth in arabinose appeared to attenuate EPEC binding to HeLa cells (Section 3.2, Figure 3.7), the effect of growth in different carbon source on *bfpA* and *perA* reporter activity was analyzed to determine whether the attenuated binding was due to an effect on expression of the BFP, which are the main adhesins involved in initial adherence. E2348/69 strains carrying either *perA:lux* or *bfpA:lux* fusions were subcultured into DMEM containing 0.2% of either glucose, arabinose, sucrose, glycerol, maltose, lactose, or D-galactose. As well the strains were also subcultured into DMEM+0.2% glucose buffered with 0.1M Tris HCl pH[7.6] to examine the expression of these reporters when the bacteria were grown under optimum secretory conditions. As can be seen by Figure 4.4A, carbon source produced no major differences in *perA* activity. Carbon source had a larger effect on *bfpA* reporter activity than it did on *perA* reporter activity. Growth in 0.2% arabinose resulted in a minor decrease in *bfpA* reporter activity (1.3-fold), growth in 0.2% glycerol, maltose, lactose, or D-galactose resulted in an approximate 2-fold decrease in activity (Figure 4.4B). These results suggest the inability of EPEC grown in arabinose to adhere to HeLa cells is not due to transcriptional repression of the *bfpA* or *perA* promoters.

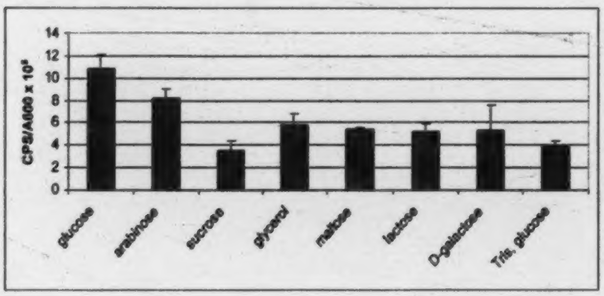
Figure 4.4. The effect of carbon source on *perA:lux* and *bfpA:lux* reporter activity in EPEC. A) E2348/69 transformed with pJW22, which encodes a *perA:lux* reporter, was diluted 1:50 from an overnight culture grown in LB at 37°C, shaking at 225 rpm, into DMEM containing 0.2% glucose, 0.2% arabinose, 0.2% sucrose, 0.2% glycerol, 0.2% maltose, 0.2% lactose, 0.2% D-galactose, or 0.2% glucose+0.1M Tris HCl pH[7.6]. Then five 200 µl samples from each culture were transferred to a 96 well plate and grown shaking at 225 rpm at 37°C for 4.5 hours before the OD_{600} and luminescence were measured. The luminescence was normalized by dividing CPS/OD_{600}. The error bars represent the standard deviation of five replicate samples taken from the same culture. The experiment was performed once.

B) E2348/69 transformed with pJW23, which encodes a *bfpA:lux* reporter, was diluted 1:50 from an overnight culture grown in LB at 37°C, shaking at 225 rpm, into DMEM containing 0.2% glucose, 0.2% arabinose, 0.2% sucrose, 0.2% glycerol, 0.2% maltose, 0.2% lactose, 0.2% D-galactose, or 0.2% glucose+0.1M Tris HCl pH[7.6]. Then five 200 µl samples from each culture were transferred to a 96 well plate and grown shaking at 225 rpm at 37°C for 4.5 hours before the OD_{600} and luminescence were measured. The luminescence was normalized by dividing CPS/OD_{600}. The error bars represent the standard deviation of five replicate samples taken from the same culture. The experiment was performed once.

A


CPS/A600 x 10^3
0
2
4
6
8
10
12
14
16
glucose
arabinose
sucrose
glycerol
maltose
lactose
D-galactose
Tris, glucose

B


CPS/A600 x 10^3
0
2
4
6
8
10
12
14
glucose
arabinose
sucrose
glycerol
maltose
lactose
D-galactose
Tris, glucose

5. DISCUSSION

Studies in uropathogenic *E. coli* have illustrated the importance of the Cpx response in the proper formation of P pili and also tantalizingly suggested that the Cpx response may be involved in regulation of virulence in this organism due to the presence of putative CpxR-P binding sites upstream of genes encoded on a pathogenicity island (Hung *et al.* 2000). It is attractive to suggest that the Cpx response may be involved in either the formation or the regulation of envelope associated virulence factors in other pathogens as well. Since EPEC requires the proper assembly and timing of expression of a number of envelope associated virulence determinants, namely the bundle-forming pili and type III secretory apparatus, it was chosen as a model organism for this study. Furthermore, preliminary results had demonstrated that the Cpx response was required for the proper formation of BFP in an MC4100 background and that an EPEC *cpxR* mutation reduced the localized adherence of EPEC bacteria to HeLa cells (A.Z. Nevesinjac and T.L. Raivio, unpublished data). Since the Cpx pathway appeared to have effects at either the translational or post-translational level, this study commenced by examining both the secretion and functionality of EPEC virulence determinants in the presence and absence of Cpx activation.

5.1 Effect of Cpx-activation on type III secretion

The construction of the type III secretory apparatus and secretion of virulence effectors are a major outcome of the second stage of EPEC pathogenesis. As it is energetically expensive to construct the secretory apparatus and translocate effectors, TTSS assembly and secretion are tightly regulated and responsive to numerous environmental cues which, *in vitro*, have been demonstrated to mimic the conditions present in the gastric tract (Kenny *et al.*, 1997b). Buffering capacity of the growth media, a temperature of 37°C, a pH of 7, $Fe(NO_3)_3$, the presence of calcium and $NaHCO_3$ as well as the absence of NH_4Cl are required for maximal EPEC secretion of EspA, B, D, 39Kda, and 40Kda proteins (Kenny *et al.*, 1997b). Although the identities of the 39 KDa and 40 KDa proteins have not been determined, they have the same mass as NleB and NleC, two recently identified, weakly secreted *Citrobacter rodentium* virulence effectors, the predicted nucleic acid sequence of which have homologues in EPEC (Deng *et al.*, 2004). Optimal buffering conditions for virulence effector secretion are provided by growth in DMEM, under 5% CO_2, though the CO_2 buffering capacity can be replaced with 0.1M Tris-HCl or 0.1M HEPES (Kenny *et al.*, 1997b)

In DMEM medium containing 0.2% arabinose, thus buffered by only the 44mM sodium bicarbonate normally present, the secretion of the *LEE4* encoded effector EspB and *LEE5* encoded effector Tir are increased through Cpx activation, whether it be through NlpE-mediated activation of the wild-type pathway or through overexpression of the response regulator CpxR (Figure 3.5). Cpx pathway activation by the same means also resulted in increased secretion of Tir and EspB in the presence of 0.1M Tris-HCl, pH 7.6 (Figures 3.3, 3.4). Thus, the Cpx pathway is able to activate secretion of EPEC virulence determinants regardless of buffering. In the absence of Cpx activation no significant EspB or Tir secretion can be detected through western analysis or ELISA of TCA precipitated culture supernatants, which is not surprising since growth in unbuffered DMEM has been shown to result in minimal secretion (Kenny *et al.*, 1997b). Cpx activation during growth in LB did not result in any detectable secretion (data not shown). This is not surprising since growth in LB has not been shown to instigate secretion unless the bacteria were transformed with the multicopy plasmid pCVD442 that carries the *per* regulon, which activates expression of *ler*, in turn increasing expression of the LEE operons (Kenny *et al.*, 1997b). This likely reflects the fact that the critical *per* and *ler* loci are not expressed under these conditions. Since data presented here indicate that the Cpx pathway may exert an effect on LEE gene expression via Per or Ler (Table 4.4) it would be interesting to see whether Cpx activation in E2348/69 (pCVD442) cells, in which the Per regulators are artificially expressed at high levels, resulted in increased secretion during growth in LB.

Once it became apparent that Cpx activation could increase EPEC secretion, the next question to be addressed was what mechanism was responsible. Several possibilities exist. For example, Cpx pathway activation could lead to increased expression of the secreted effectors or the genes encoding the TTSS. Alternatively, perhaps Cpx-activated protein folding and degrading factors function to increase assembly of the TTS apparatus or enhance folding of the secreted effectors.

To test whether type III secretion (TTS) functioned more efficiently when the Cpx response was artificially activated, hemolysis assays were performed. The functionality of type III secretory systems in EPEC as well as pathogens such as *S. flexneri* and *Y. enterocolitica* has been demonstrated through hemolysis of sheep red blood cells (Warawa *et al.*, 1999; Neyt and Cornelis, 1999; Blocker *et al.*, 1999). Bacteria encoding TTSS are able to translocate effector molecules into RBC, causing hemolysis. This hemolysis is contact dependant in that it requires the bacteria to be centrifuged onto the blood cells for delivery of type III secreted effectors into the erythrocyte membrane. The

EspB and EspD proteins of EPEC, responsible for the formation of the translocation pore in the erythrocyte membrane, have homology to the *Yersinia* YopB and *Shigella* IpaB proteins, which are also responsible for the formation of pores in eukaryotic membranes (Ide *et al.*, 2001, Warawa *et al.*, 1999; High et al., 1992; Håkansson et al.,1996; Tardy et al., 1999; Wachter *et al.*, 1999). Therefore, it was predicted that since Cpx activation increased secretion of EspB, and presumably EspD, as it lays in the same operon as *espB*, an increase in contact dependent hemolysis would be observed when either *cpxR* or *nlpE* overexpression was used to upregulate the Cpx response in EPEC. This was proven to be the case, though with a caveat. Wildtype EPEC, when grown statically at 37°C in 5% CO_2 to an OD_{600} of 0.7, lyse approximately 15% of the red blood cells in a 4% red blood cell-DMEM solution (Warawa *et al.*, 1999). This lysis was contact dependent as significant hemolysis was not observed when the blood cells were incubated with cell free culture supernatant or were separated from the bacteria with a 0.4μm size filter, which allows diffusion of proteins but prevents bacteria-red blood cell contact (Warawa *et al.*, 1999). To detect a difference in hemolysis between Cpx-activated and non-activated backgrounds, cells at an optical density of 0.5 had to be used; at higher densities the hemolysis was equal. At this OD_{600}, Cpx pathway activation by *nlpE* or *cpxR* overexpression lead to a 2.6-3.7-fold increase in hemolysis (Figure 3.2). This correlates with the transcriptional data as the Cpx effect on pathogenicity genes is evident between an OD_{600} of 0.2 and 0.5 and then falls to vector control levels by an OD_{600} of 0.6-0.8. However, the maximum level of hemolysis detected was approximately 4%, and even when the bacteria were grown to an OD_{600} of 0.7 the reported hemolysis level of 15% was not obtained (Figure 3.2). One potential explanation for this reduction in contact-dependent hemolysis was provided by the pedestal assay experiments. While attempting to assay the effect of Cpx pathway activation on pedestal formation, it was observed that growth in arabinose to induce *cpxR* or *nlpE* overexpression inhibited microcolony formation. Thus, contact dependent hemolysis may have been unlikely under the conditions employed as it appeared few EPEC cells were probably able to stay bound to the erythrocytes to deliver EspB and EspD into the membrane. This explanation suggests that BFP are required to adhere to blood cell membranes, a contention that could be examined using JPN15, which lacks the BFP encoding EAF plasmid, in hemolysis assays. It would be interesting to see whether increases in contact dependent hemolysis could be observed if the Cpx system was activated through *nlpE* or *cpxR* overexpression using either constitutive expression via a pUC19 or pBR322-based vector, or through a different inducible vector such as the IPTG-inducible vector pTRC99.

5.2 Effect of sugar and Cpx-activation on BFP expression

The observation that arabinose inhibits EPEC adherence to Hela cells may potentially lead to insights about either regulation of the *bfp* genes or BFP interaction with the host cell membrane. The effect is quite rapid as EPEC grown in DMEM+0.2% glucose, a condition conducive to adherence, then centrifuged onto Hela cells to initiate infection in the presence of DMEM+0.2% arabinose, exhibits attenuated binding after only 40 minutes of incubation in the presence of arabinose (Figure 3.6). Interestingly, previous studies have demonstrated that galactose reduces EPEC adherence to HEp-2 cells to levels observed with JPN15, which lacks the EAF plasmid and is basically non-adherent (Van maele and Armstrong, 1997). Since growth in galactose reduced the levels of expressed BfpA to JPN15 levels, the transcriptional activity of the *perA* and *bfpA* promoters was assayed in glucose, arabinose, maltose, sucrose, glycerol, lactose, and galactose to determine if some sugars might inhibit transcription of the *bfp* locus. The *bfpA* fusion showed slightly lowered activity in arabinose and between a 2 and 2.5 fold decrease in the remaining sugars, while the *perA* reporter showed a minor reduction (less than 1.5 fold) in activity in lactose and D-galactose (Figure 4.4). However, neither reporter showed enough inhibition to suggest that the reduced adherence observed in arabinose and galactose was due to a repression of transcription. Hence, the effect was likely post-translational due to its rapidity, though a translational effect cannot be ruled out. It is possible that sugars could modulate *bfp* or *per* transcription through a regulatory element located upstream of the DNA included in the promoter fusion. This could be addressed through RT-PCR analysis of *bfpA* transcripts in RNA harvested from cells grown in glucose, galactose and arabinose. The reduction in microcolony formation could also be due to the transcriptional activation of *bfpF*, the product of which has been implicated in the conversion of BFP from thin to thick filaments associated with pilus retraction and subsequent dispersal of the microcolony (Knutton *et al.*, 1999). Similarily this could be determined through RT-PCR analysis of *bfpF* transcription, which could allow the dissection of the signals involved in activating dispersal, at least on a transcriptional level. BfpA immunoblots could be performed on EPEC strains grown in glucose before and after a shift to arabinose containing media. A large decrease in BfpA protein would support the idea of a post-transcriptional effect if no major change in *bfpA* or *perA* transcription is observed during such a shift.

5.3 Generation and analysis of *luxCDABE* promoter constructs

Since Cpx activation had such a potent effect on TTS and the secretion of EspB and Tir, the next question was to determine at what level this effect occurred. Several possibilities exist. For example, Cpx pathway activation could lead to increased expression of the secreted effectors or the genes encoding the TTSS. Alternatively, perhaps Cpx-activated protein folding and degrading factors function to increase assembly of the TTS apparatus or enhance folding of the secreted effectors. To determine if the Cpx response affected transcription of the virulence genes of EPEC, *lux* reporters were constructed to monitor expression of the LEE pathogenicity island genes.

To date, no low-copy fluorescent or luminescent promoter-probe vectors have been employed to analyze gene expression in EPEC. The majority of reporters are high copy chloramphenicol-acetyl transferase or *gfp* reporters (Ibarra *et al.*, 2003; Umanski *et al.*, 2002); few *lacZ* reporters are used as the native EPEC *lacZ* gene in EPEC makes it difficult to use these fusions due to high background β-galactosidase activity. A solitary chromosomal *LEE4:lacZ* fusion has also been described, but the fact that only one chromosomal reporter has been described in the literature attests to the difficulty and laborious procedure used to construct these strains (Grant *et al*, 2003). An E2348/69 Δ*lacZ* strain has been created, but no data have been published using reporter plasmids in this background. The advantage to using *luxABCDE* or *gfp* reporters is that real time data can be collected from promoter fusions in live bacteria as the cells do not have to be lysed to obtain data. This makes the *lux* and *gfp* system very conducive to high throughput assays and dissection of multi-step regulation cascades as evidenced by analysis of the flagellar gene expression cascade (Kalir *et al.*, 2001).

5.3.1 Analysis of *lux* and *lacZ* reporters in MC4100

While the *lacZ* and *lux* fusions in MC4100 *cpxR:spc* reflected the same general trend with respect to the effect of Cpx activation, there were notable differences. Both *LEE2* and *LEE3* reporter activity increased in response to CpxR overexpression using both types of reporters, but the *lux* fusions tended to be much more sensitive. *LEE2* and *LEE3* had 445-643 and 36-84 fold inductions, respectively, using the *lux* reporters, while the *lacZ* reporters gave values of 6.8 and 5 fold, respectively (Table 4.1, Figure 4.1). The *cpxR:lux* fusion also had a high level of induction, 5-8 fold, relative to a *cpxR:lacZ* fusion, which has been demonstrated to increase 2-4 fold in response to Cpx activation (Table 4.1; DiGiuseppe and Silhavy, 2003). The Cpx-regulated *degP, dsbA, spy,* and *ppiA* promoter fusions all have similar levels of induction, between 2-4 fold, in response to Cpx activation (DiGiuseppe and Silhavy, 2003). A *cpxP:lacZ* fusion, the promoter most sensitive to Cpx activation, increased 15 fold after exposure to elevated pH, a Cpx-activating signal (DiGiuseppe and Silhavy, 2003). Thus, the changes in *LEE2* and *LEE3* expression seen in response to overexpression of CpxR in MC4100 are very large relative to the induction ratios seen for previously studied Cpx-regulated genes. No major difference was observed in *LEE4* expression in response to CpxR overexpression using *lux* or *lacZ* reporters (Table 4.1; Figure 4.1). Although an induction between 36 and 600 fold seems very large, it is difficult to ascertain the relative strength of the Cpx effect on LEE2 and LEE3 promoters using *lux* fusions without a stronger positive control, such as a *cpxP:lux* fusion. Other possible explanations for the discrepancies between the induction ratios seen in this study and those previously reported for other Cpx-regulated genes (Otto and Silhavy, 2002; DiGiuseppe and Silhavy, 2003) include the increased sensitivity of the lux reporters, and the copy number of the reporter vector. As well, the method of induction is another possible explanation for the discrepancy. CpxR overexpression is an artifical means of activating the Cpx pathway as overexpressing the response regulator bypasses CpxA.

The activity of the *perA* fusion, the promoter of which controls expression of the *perABC* operon, increased 12-14 fold in response to CpxR overexpression in MC4100, higher than the 5-8 fold increase seen for the *cpxR* promoter, which is a weakly up-regulated member of the Cpx regulon (Table 4.1). Given the large induction ratios observed for the *LEE2* and *LEE3* reporters under the same conditions, the results obtained with the *perA* fusion in the MC4100 background suggested a weak Cpx-dependent activation of the promoter. As Per is a regulator of Ler, which in turn increases expression of *LEE2, LEE3, LEE4*, and *LEE5*, increases in levels of Per would be expected to have huge downstream consequences. Thus, this relatively small increase in *perA:lux* reporter activity seen in response to CpxR overexpression may be physiologically significant.

LEE1 expression increased approximately 25 fold in response to CpxR overexpression in MC4100 as measured with the *lux* reporter, however no change was detected using the *lacZ* reporter, or through RT-PCR in EPEC. As the *lux* reporters are more sensitive than the *lacZ* reporters, this may reflect a minor increase in transcription in response to Cpx activation that is only detectable using the *lux* reporter. As the *perA:lux* induction was even lower, 12-14 fold, one might expect that a *perA:lacZ* reporter might also show no induction in response to Cpx activation.

The *LEE1* and *LEE4:lacZ* fusions had reduced activity in a *cpxR:spc* mutant compared to wild-type MC4100. Curiously the overexpression of CpxR did not affect expression of these reporters. This may be explained if *LEE1* and *LEE4* expression is dependent on CpxA. As *cpxA* is downstream of *cpxR*, an insertional inactivation of *cpxR* likely has polar effects on *cpxA*, which is part of an operon with *cpxR*. Similarily, the expression of *hilA*, which encodes a *Salmonella enterica* serovar Typhimurium virulence regulator, has recently been shown to be dependent on CpxA, but not CpxR (Nakayama *et al.*, 2003).

This data suggested two possible mechanisms for the Cpx effect on secretion. The first mechanism is a Cpx-dependent activation of *perABC*, with Per activating Ler, which could then subsequently activate the expression of the remaining operons. However, as the induction of *perA* was on the level of *cpxR*, a weakly Cpx-regulated gene, this may not be likely. The other possibility suggested by the fusions in MC4100 was that the Cpx pathway upregulated the expression of the *LEE2* and *LEE3* operons, which encode the type III secretory system. Increased production of the TTSS would allow for the secretion of more effectors. Together, the *lacZ* and *lux* fusions in MC4100 show increases in *LEE2* and *LEE3* expression in response to Cpx activation, while the *lux* reporters alone showed increases in *perA* and *LEE1* activity.

5.3.2 Construction of an EPEC compatible Lux reporter plasmid

Once data had been obtained using the pNLP10 based reporter fusions in MC4100 an attempt was made to introduce the plasmids into EPEC. Except for solitary colonies produced by transformation attempts with pNLP10, *cpxR:lux* in pNLP10 and *cpxP:lux* in pNLP10, all transformation attempts failed. As this was initially thought to be a restriction problem, a variety of temporary restriction alleviation methods were tried, including heat inactivation and UV irradiation, all of which failed to allow pNLP10-based vectors to be transformed into EPEC. Some evidence suggested that the problem was not restriction related but perhaps an origin incompatibility. While trying to insertionally inactivate the *cpxR* gene using the lamba *red* recombinase system residing on a pSC101 origin-containing plasmid (pKD46) an attempt was made to introduce the pKD46 plasmid into an E2348/69 background. The plasmid proved impossible to introduce into EPEC and considering that, of the pNLP10 based vectors introduced to EPEC, only a single colony was ever produced, this suggested that the pSC101 origin may be incompatible with EPEC. A search of the literature revealed that pACYC184-based vectors, which carry the low-copy p15a origin, are commonly used in EPEC (Gómez-Duarte and Kaper; 1995; Grant *et al.*, 2003), thus the pSC101 origin was removed from pNLP10 through *Pac*I digestion and the p15a origin was PCR amplified using *Pac*I tagged primers, digested, and cloned into digested pNLP10 to produce pJW15. Initial attempts to introduce pJW15 based vectors into EPEC again proved unsuccessful. However since pACYC184 is stable in EPEC it was likely due to restriction as opposed to origin incompatibility. A protocol using a thirty minute 50°C incubation during preparation of competent cells, grown to an OD_{600} of between 0.4 and 0.6, to temporarily inactivate the restriction system proved successful, allowing consistent introduction of pJW15-based reporters into EPEC (Gonzalez *et al.*, 1999).

5.3.3 Analysis of the effect of Cpx pathway activation on virulence gene expression in EPEC

The promoter regions of *cpxR, bfpA, perA, LEE2, LEE3,* and *LEE4* were cloned into pJW15. During the course of this study, it was found that plasmids isolated from an EPEC background were much easier to subsequently transform into other EPEC backgrounds, presumably because the first transformation allowed modification of the plasmid by any existing restriction/modification systems. Thus, the plasmids were first transformed into E2348/69 and then subsequently reisolated from EPEC and transformed into E2348/69 carrying the vector control, pBAD18, the *nlpE* overexpression plasmid, pND18, and the *cpxR* overexpression plasmid pROX.

The *perA:lux* reporter in MC4100 had a 12-14 fold induction in response to Cpx activation, an induction higher than that of *cpxR* (Table 4.1). However, its induction was much lower than the 34-640 fold induction observed for *LEE2* and *LEE3* in MC4100 under the same conditions (Table 4.1). While the MC4100 data was partially suggestive of Cpx regulation through the Per-Ler cascade, the EPEC data fails to corroborate this. The RT-PCR analysis of *perA* and *ler* failed to detect differences in expression in response to CpxR overexpression in an E2348/69 *cpxR:kn* background (Figure 4.1). The lack of CpxR in this strain would make the RT-PCR analysis more sensitive by reducing basal expression of Cpx-regulated genes, increasing the induction when CpxR was overexpressed. NlpE and CpxR overexpression in E2348/69 grown in DMEM resulted in 1.4 and 1.6-2.1 fold inductions of the *perA* fusion, respectively (Table 4.4). When the bacteria were grown in LB, NlpE and CpxR caused .9-1.0 and 2.13-2.5 fold inductions, respectively (Table 4.4). This is slightly higher than the 1.5 and 1.6 fold inductions of *cpxR* expression that was seen when the bacteria were grown in DMEM and LB, respectively, in response to CpxR overexpression (Table 4.3). Since the effect of the Cpx response on *perA* expression in EPEC was relatively small (1.4-2.1 fold), it may be that it is insignificant and that the actual transcript levels do not change in response to activation of the Cpx response. The low induction seen using *lux* reporters and failure to detect differences in *perA* expression in response to CpxR overexpression through RT-PCR analysis suggests that the observed effect of the Cpx response on EPEC pathogenicity gene expression is not mediated through increased *perA* expression.

As with the RT-PCR examining *perA* transcript levels, *ler* transcripts were difficult to consistently amplify using RT-PCR. Sometimes an increase in *ler* transcripts could be detected in response to *cpxR* overexpression, however unlike the RT-PCR analysis of the *LEE2*, *LEE3*, *LEE4* and *tir* operons, the *ler* RT-PCR was never sharp and almost always had a diffuse smear under the RT-PCR product. The *ler* gene may also be regulated by transcript stability. The difficulty in amplifying *LEE1* transcript may reflect this transcript stability. Again, this could be verified through transcript half-life analysis. A *LEE1* fusion currently being constructed may also give insight into transcriptional regulation of *ler*. The RT-PCR data suggests that in EPEC Cpx activation does not have a major effect on LEE1 expression.

The analysis of virulence gene expression in the presence of a *ler* deletion yielded expected results, a decrease in expression of the Ler-regulated *LEE3* fusion (Table 4.4). However, analysis of *perA* and *bfpA* reporters in the same background produced some surprising results suggestive of an inhibitory effect of Ler on *perA* and *bfpA* expression. The observation that the *perA* and *bfpA* fusions are 5-10 fold more active in E2348/69

Δler compared to wild-type EPEC is suggestive of a negative feedback loop between Per and Ler (Table 4.4). The 1.6-2.1-fold induction of *perA:lux* expression in response to CpxR overexpression is undetectable in the absence of Ler, perhaps because some form of Ler repression has been alleviated and the promoters are now expressed to such high levels that Cpx activation is obscured. Alternatively, the Cpx effect may be so small as to be insignificant. Ler repression of *perA* is logical from a pathogenicity perspective. Signals in the intestine would cause an activation of *perA*, the expression of which would in turn increase BFP levels, mediating adherence. Increased levels of Per would activate expression from *LEE1*, and Ler then could proceed to instigate formation of the type III secretory apparatus and secretion of effectors. As BFP would be undesirable for intimate attachment, since the pili could prevent enterocytes from getting close enough for Tir-Intimin interaction, once Ler reached a certain cellular level it could inhibit *perA* expression, providing an additional level of regulation for the expression of pathogenicity genes. This hypothesis could be confirmed by examining *perA* and *bfpA* expression in the presence and absence of Ler overexpression.

The *bfpA* fusion essentially had vector control levels of expression in MC4100, but was highly active in EPEC which demonstrates the dependence on the presence of the Per regulatory proteins for the activity of this promoter. The promoter activity increased in response to *nlpE* and *cpxR* overexpression between 1.5 and 2.3 fold (Table 4.3). RT-PCR must still be performed to determine whether or not increased *bfpA* promoter activity leads to increased levels of *bfpA* transcript. As well, BfpA levels in E2348/69 carrying the vector control, pBAD18, and the *cpxR* overexpression plasmid, pROX should be examined using either immunoblotting or ELISA. As the *bfpA* fusion was essentially inactive in MC4100 it is probable that the Cpx effect was not direct and likely mediated through *perA*, the only known activator of *bfpA* expression.

The *LEE2* and *LEE3* promoter fusions demonstrated large levels of induction when CpxR was overexpressed in MC4100. Similar to *perA*, a strong induction in response to Cpx activation was not observed in EPEC. When NlpE and CpxR were overexpressed in an EPEC background in DMEM, the *LEE2* reporter activity increased 1.5 and 1.8-2.3 fold respectively (Table 4.3). The *LEE3* reporter was induced 2.7-3 fold in response to NlpE and CpxR overexpression in DMEM (Table 4.3). Although this is higher than the level of induction observed for *cpxR* , the increase in expression is still much lower than the 36-660 fold induction observed in MC4100 (Table 4.1). This may be due to EPEC-specific regulatory factors such as Ler and PerC, which elevate expression of virulence determinant genes, and consequently may overwhelm any observable Cpx effect on these operons. When Ler is present but Per is absent, the Cpx-mediated increase in *LEE3* expression is not observed as the reporter expression does not change in response to CpxR overexpression in JPN15 compared to the 2.7-3 fold induction observed in E2348/69 (Tables 4.3, 4.4). This suggests that the small amount of activation of *LEE3* expression by CpxR or NlpE overexpression requires the presence of Per. NlpE and CpxR overexpression in LB caused a 0.5-0.8 fold reduction in expression of the *LEE2* reporter while causing a 0.5 and a 1.8 fold change in *LEE3* expression, respectively (Table 4.3). The decreased expression of the *LEE2* reporter was curious as CpxR overexpression in LB caused increases in both *LEE2* and *LEE3* expression in RT-PCR analysis (Figure 4.1). However, the RT-PCR analysis was performed on RNA from E2348/69 *cpxR:kn* cells and the lack of CpxR might make small Cpx-dependent differences more detectable. Thus, cumulatively, the RT-PCR and *lux* reporter data suggest that the Cpx system has a minor effect on *LEE2* and *LEE3* expression in an EPEC strain background.

The *LEE4* operon, the expression of which is only moderately increased by Ler (Mellies *et al.*, 1999), exhibits only a slight increase (1.6 fold) in activity in response to CpxR overexpression, but a larger increase (3.2-4 fold) in response to NlpE overexpression in an EPEC background (Table 4.3). Similarly, in MC4100, overexpression of CpxR yielded only a 1.9-2.1 fold increase in expression of the *LEE4-lux* fusion (Table 4.1) As well, RT-PCR analysis failed to show any Cpx-dependent increases in *espA* transcript levels (Figure 4.1), a *LEE4*-encoded gene. Thus, the marked difference in secretion of EspB observed in E2348/69 (pROX) compared to E2348/69 (pBAD18) was not likely due to increased transcription of *LEE4*, but could reflect the increase in expression of *LEE2* and *LEE3*, which would allow for more type III secretory system components to be made and thus permit more secretion. Alternatively the majority of the effect could be post-transcriptional, with Cpx-regulated folding factors increasing levels or efficiency of the TTSS, therefore increasing secretion of virulence determinants.

5.4 NlpE overexpression-mediated effects on secretion and reporter activity

The use of *nlpE* overexpression as a Cpx activating cue is important as it activates the wild-type Cpx pathway as opposed to *cpxR* overexpression, an artifical activation cue. None-the-less, both approaches appear to be valid. Both CpxR and NlpE overexpression cause increased hemolysis and secretion of EspB and Tir (Figures 3.2, 3.4, 3.5). Thus, this evidence suggests that CpxR overexpression is a valid, physiologically significant way of detecting Cpx-activated changes in pathogenicity gene expression.

However, some differences were observed when the effects of NlpE vs CpxR overexpression on *LEE4-lux* activity in EPEC were compared (Table 4.3). The *LEE4* fusion was the only reporter that had a higher activity when the Cpx pathway was activated through NlpE overexpression (3.2-4 fold) as opposed to CpxR overexpression (1.6-1.7 fold) in EPEC grown in DMEM (Table 4.3). Curiously, when NlpE is overexpressed, an increase in secretion of *LEE4*-encoded proteins is detected, a regulatory mechanism which appears to be CpxR independent. Sypro Ruby staining of secreted effector proteins suggests that *nlpE* overexpression in an E2348/69 *cpxR:kn* strain background still causes an increase in EspB secretion at an OD_{600} of 0.5, while at this time point a lesser increase is observed in response to *cpxR* overexpression and no secretion is observed in the vector control carrying strain (Figure 3.8). This suggests an additional level of complexity to the involvement of the Cpx response in EPEC virulence determinant regulation. A recent study has demonstrated that expression of *hilA*, an activator of genes involved in host cell invasion by *Salmonella enterica* serovar Typhimurium, is severely attenuated in a *cpxA* mutant with a corresponding decrease in SipA, an invasion gene product, and decreased ability to invade epithelial cells (Nakayama *et al.*, 2003). Surprisingly a *cpxR* mutant showed markedly less attenuation of *hilA* expression than the *cpxA* mutant and had no significant reduction in either SipC levels or invasion ability (Nakayama *et al.*, 2003). In this study, since the *cpxR:kn* insertional mutation likely has polar effects on *cpxA*, a *cpxA* knockout would be a valuable tool in determining how this NlpE-dependent, CpxR-independent signal is transduced.

5.5 The role of Cpx activation in EPEC pathogenesis

During the course of a successful infection, a bacterium must regulate expression of its virulence determinants both temporally and spatially. EPEC must time the production and dispersal of bundle-forming pili to coincide with the presence of host cells, then properly assemble a type III secretory apparatus and deliver effectors into the host cell. Numerous transcriptional regulatory systems and their associated regulatory cues have been characterized in EPEC. A quorum sensing system activates *LEE1* and *LEE2* expression directly and enhances the expression of *per* indirectly through an as yet uncharacterized regulatory protein (Sperandio *et al.*, 1999). The GadX protein is activated in response to an external acidic pH and represses *perA* expression, presumably preventing virulence determinant expression until the bacterium has passed from the acidic environment of the stomach to the alkaline environment of the intestine (Shin *et al.*, 2001). The nucleoid-like proteins also have a role in transcriptional regulation. Fis inhibits BFP expression and activates *LEE1* and *LEE4* expression in early exponential phase (Goldberg *et al.*, 2001). H-NS repression of the *LEE2, LEE3,* and *tir* promoters is overcome by Ler binding (Bustamente *et* al., 2001, Sanchez –SanMartin *et al.*, 2001). H-NS also inhibits transcription of *LEE1* at 27°C, a repression that is alleviated at 37°C (Umanski *et al.*, 2002). Finally, IHF activates expression of *LEE1* and mediates repression of the flagellar transcriptional activator *flhDC* (Friedberg *et al.*, 1999; Yona-Nadler *et al.*, 2003). Additionally, the recently characterized BipA GTPase is believed to enhance *LEE1* expression through translational regulation of a yet undetermined regulator (Grant *et al.*, 2003).

Based on the EPEC reporter and RT-PCR data, the effect of CpxR activation on EPEC secretion appears to be predominantly post-transcriptional. It seems likely that an increase in Cpx-regulated protein folding and degrading factors aids in the proper assembly of the TTSS. There appears to be a minor transcriptional effect as well. The fact that no significant increase in *LEE3* expression occurs when CpxR is overexpressed in JPN15, a strain which lacks the EAF plasmid carrying the Per regulator, supports this hypothesis as does the fact that in MC4100 *perA-lux* is induced 12-14 fold by CpxR overexpression. The lack of a strong induction in response to NlpE or CpxR overexpression of the *bfpA* and *perA* reporters in EPEC weakens this hypothesis, as does the lack of a Cpx-dependent difference in *perA* transcript abundance in RT-PCR.

The MC4100 reporter data hints at an increased level of complexity. Of the previously mentioned regulatory pathways, only the transcriptional patterns observed through alleviation of H-NS repression fit the transcriptional profile of *LEE* operons in the MC4100 Cpx-activated background. In MC4100 overexpression of *cpxR* results in activation of *perA LEE1, LEE2, LEE3* expression, the four operons which have been demonstrated to be H-NS repressed. It is possible that CpxR-P interacts with the promoters of these operons and either prevents H-NS from binding or binds and stimulates the release of H-NS. However, as mentioned, it is curious that in an EPEC background *LEE1* shows no significant difference in expression in the presence of Cpx activation. H-NS binds to intrinsically curved DNA and the curvature of the DNA can be predicted using the model.it program available at http://www.icgeb.org/~netsrv/.

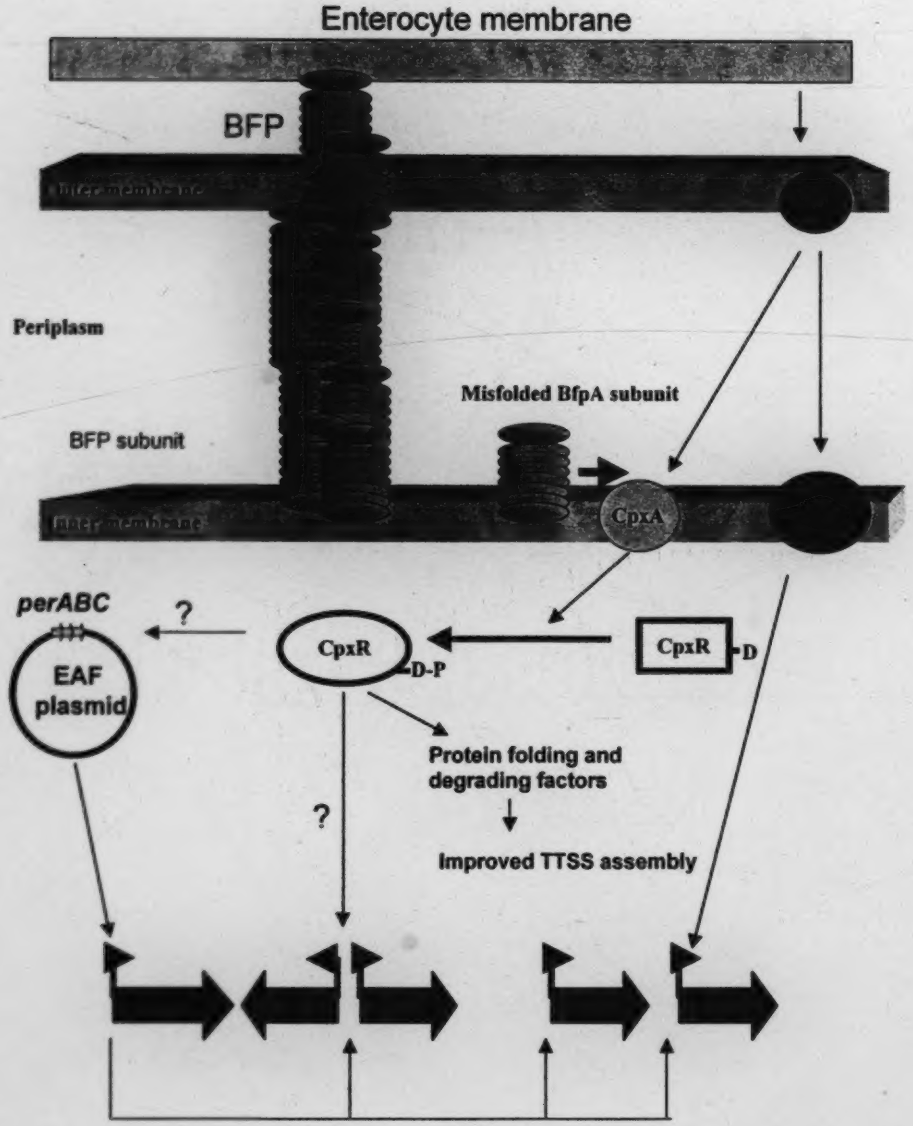
Analysis of the intergenic region between *cpxRA* and *cpxP* containing the divergent promoters has revealed an area predicted to have a high level of curvature over a range of 100 basepairs with five curvature peaks. This curved area is conserved in the *cpxP/cpxRA* promoter region in *E. coli* strains MC4100, 0157:H7 (EHEC), as well as *Shigella flexneri* and *Salmonella typhimurium*. Since this region has more curvature than the H-NS binding sites in the *LEE/LEE3* intergenic region and the *tir* promoter, it is possible that H-NS would bind to and repress the promoter *in vivo*.. Thus, perhaps alleviation of H-NS repression is part of the mechanism of transcription activation employed by CpxR~P. Whether H-NS can bind the *cpxP/cpxRA* promoter fragments could be assayed through an electrophoretic mobility shift assay. If indeed the fragment binds H-NS, the *in vivo* effect of H-NS on the activity of *cpxP* and *cpxRA* expression could be assayed through fusions of the promoters to the *luxABCDE* reporter in an H-NS mutant background. *ler* is an H-NS repressed gene whose product functions as an H-NS antagonist for its target promoters. Similarily, CpxR could also be an H-NS repressed regulator that functions as an H-NS antagonist at H-NS repressed target promoters. Further support for the idea that the CpxR and H-NS regulatory mechanisms may be connected derives from recent studies which demonstrated that *dsbA*, a Cpx-regulated gene important for virulence in a number of pathogens, is H-NS repressed during exponential growth in *Salmonella enterica* serovar Typhimurium (Gallant *et al.*, 2004). The *dsbA* promoter region in *Salmonella enterica* serovar Typhimurium has several curved areas over 200 base pairs, while the *Shigella flexneri dsbA* has several prominent curves over 200 basepairs. As well, the *virF* gene in *Shigella*, which is a Cpx-activated master regulator that controls a regulatory cascade resulting in the expression of several operons involved in invasion of epithelial cells, has been shown to be repressed by H-NS, while alleviation of this repression occurs at temperatures above 32°C (Nakayama and Watanabe, 1998; Prosseda *et al.*, 2004). As H-NS is a key regulator in EPEC pathogenicity, it would be very interesting to examine whether a relationship exists between the Cpx two-component system and H-NS regulation.

The work to this point describes the nature of the Cpx regulatory system's involvement in regulation of EPEC pathogenicity genes but does not address its potential role *in vivo* during an infection. The fact that Cpx activation increases transcription of the *LEE2, LEE3,* and *LEE5* operons as well as secretion of effectors encoded by the *LEE4* and *LEE5* operons suggests that one role of the Cpx response could be in the second stage of pathogenesis, assembly of the type III secretory system and the secretion of effectors that lead to the intimate attachment characteristic of the third stage of infection. It has been noted that as microcolonies disperse, the bundle forming pili, which are very hydrophobic envelope associated virulence determinants, undergo a morphology transformation where the pili are transformed from thin to thick filaments (Knutton *et al.*, 1999). The dispersion protein *bfpF* has homology to *P. aeruginosa* PilT, which is an ATPase that powers retraction of type IV pili in this organism (Anantha *et al.*, 1998). It is possible that pilus retraction may cause a form of periplasmic stress, perhaps the aggregation of pilus subunits in the inner membrane, which are removed from the retracting pilus. This would activate the Cpx system through CpxA directly, bypassing NlpE (Figure 5.1). Alternatively, host cell contact as a result of pilus retraction could activate the pathway. It has recently been demonstrated that outer membrane interaction with a hydrophobic surface can cause Cpx pathway activation that is mediated through the NlpE protein (Otto and Silhavy, 2002).

The activation of EPEC pathogenesis described in this thesis may be separated into two stages. The first is NlpE-independent and may involve CpxA sensing misfolded BfpA subunits that accumulate in the inner membrane as a result of pilus retraction. This could result in an increase in the TTSS, either transcriptionally through *perA*, or post-transcriptionally, through Cpx-regulated folding factors that might aid in the assembly of the TTSS. Increased levels of the TTSS would allow for increased secretion of virulence determinants. In the second stage, which is NlpE dependent, contact with the hydrophobic host cell would lead to further stimulation of the Cpx response and secretion of LEE4 virulence determinants. In support of this idea, NlpE overexpression was demonstrated to increase activity of a *LEE4* reporter fusion. This stage of virulence determinant expression may be partly or totally Cpx independent, since NlpE overexpression in a E2348/69 *cpxR:kn* background results in increased secretion of EspB (Figure 3.6). Thus Cpx activation through pilus subunit accumulation, which is CpxR dependent, increases levels of the TTSS, while contact with the host cell membrane results in a CpxR independent NlpE signal resulting in increased secretion of LEE4 virulence determinants.

During the course of an infection, Cpx activation could serve as a cue for the cell indicating that the microcolony has dispersed and it is an appropriate time to activate expression of type III secretory genes. As the Cpx system has been demonstrated to inhibit motility in E2348/69 as it does in MC4100 (Figure 3.8), this inhibition could be

Figure 5.1. Proposed model for Cpx regulation of EPEC pathogenesis. Upon attachment to an enterocyte EPEC bundle forming pili are unable to extend further and BfpA subunits which can no longer be added to the end of the pilus accumulate in the inner membrane and become misfolded, activating the Cpx response directly through CpxA. Alternatively, dispersal of BFP or adhesion to an enterocyte brings the bacterial outer membrane in close proximity to hydrophobic molecules, activating NlpE, which then transduces a signal, either through CpxA or another unknown regulator. CpxA autophosphorylates at its conserved histidine residue and transfers the phosphate to the aspartate residue of CpxR, which may then activate expression of *perA*. PerA can then increase *LEE1* expression and Ler can activate the remaining operons in the LEE. Alternatively, the upregulated production of Cpx-controlled folding factors may aid in folding the type III secretory apparatus. The predominant effect of Cpx activation is to increase production of the type III secretory apparatus, thus allowing more effectors to be secreted. NlpE appears to be able to activate expression of *LEE4* in a CpxR independent manner (X).


Enterocyte membrane
BFP
Outer membrane
Periplasm
Misfolded BfpA subunit
BFP subunit
Inner membrane
CpxA
perABC
?
EAF
plasmid
CpxR
D-P
CpxR
D
Protein folding and
degrading factors
?
Improved TTSS assembly

involved in the conversion of the bacterium from an avirulent planktonic stage to an amotile virulent phase (Grant *et al.*, 2003). Since flagella can function in adherence to host cells (Giron *et al.*, 2002), perhaps the inhibition of motility facilitates the binding of flagella to host cells. Once activated, the Cpx system could potentially aid in formation of a functional type III secretory apparatus through the increased expression of envelope protein folding and degrading factors. These factors might aid in the folding of periplasmic components and the degradation of misfolded aggregates. The activated Cpx system might also increase *perA* transcription. Increased Per levels could then activate *ler* expression which could in turn cause increased transcription of *LEE2, LEE3, LEE4,* and *LEE5.* Once the type III secretory apparatus was properly assembled, the NlpE cue, signaling cell adhesion, could then activate expression of the *LEE4* operon. This would then result in formation of the EspA translocation tube and an EspB, EspD pore in the enterocyte membrane, allowing for secretion of virulence effectors.

5.6 Future directions

This study opens many avenues for future research. One major question that needs to be addressed is whether the Cpx effect is transcriptional or post-transcriptional. The secretion of virulence determinants can be monitored through SDS-PAGE, immunoblotting, ELISA, and hemolysis in a variety of Cpx regulon mutant backgrounds. However, these studies will be dependant on developing an efficient technique to inactivate genes in EPEC. In particular, the disulfide bond oxidoreductase *dsbA,* the peptidyl-prolyl isomerases *ppiA* and *ppiD* and the protease *degP* should be deleted in E2348/69 and the effect on secretion should be noted. To date only a *cpxR:kn* mutant has been developed in the lab. In order to confirm the RT-PCR data from the EPEC *cpxR:kn* mutant using pJW15-based *lux* reporter fusions, mutants using different antibiotic cassettes must be constructed as the pJW15 plasmids are kanamycin resistant. This would help determine whether the Cpx effect on virulence was primarily through increased transcription of *perA* or through upregulation of folding factors that increased TTSS assembly.

The role of NlpE in EPEC virulence gene regulation is interesting. The effect of an *nlpE* null mutation on eukaryotic cell interaction using a HeLa cell model could be examined. A screen to look for genes involved in the activation of *LEE4* by *nlpE* overexpression could be performed. The E2348/69 Δ*lacZ LEE4:lacZ* reporter strain could be transformed with pND18 and, as *nlpE* overexpression increases *LEE4* expression, this should produce an observable phenotype on lactose MacConkey's indicator agar. Tn*phoA* mutagenesis could then be performed and the resulting pool of mutants could be screened for the loss of *lacZ* expression. The insertion locations of any candidate mutation could then be determined through sequencing and site directed mutagenesis in the E2348/69 Δ*lacZ LEE4:lacZ* strain could be used to confirm the Tn*phoA* mutation was responsible for the loss of NlpE-dependent *LEE4* activation. This might allow the separation of the CpxR dependent and independent signals discussed in this thesis.

All of the ideas presented in this thesis regarding the role of the Cpx pathway during the course of an infection are based on *in vitro* studies. It would be interesting to create *cpxA*, *cpxR* and *nlpE* mutations in *Citrobacter rodentium* and use these strains in a murine infection model to determine whether colonization or colonic hyperplasia were affected by the mutations. These mutants would not likely lose the ability to cause infection but would have an altered temporal expression of virulence determinants and perhaps bind to enterocytes and express pathogenicity genes at an inappropriate location in the intestine. It would be expected that the *cpxA* mutant would have more deleterious effects than either the *cpxR* or *nlpE* mutants as it seems possible that CpxA relays multiple signals to different regulators (Nakayama *et al.*, 2003). These studies would provide insight into the roles of each component of the Cpx pathway in the course of an infection.

The *lux* fusions could be valuable for examining bacterial gene expression in response to host cells. Promoter fusions to *lux* will have to be constructed for the remaining operons, *LEE1* and *tir.* The creation of the *LEE:lux* reporter fusions could conceivably allow for temporal and spatial expression of the LEE operons to be examined during the course of binding to Hela cells and intiation of pedestal formation. Similar studies could be used to determine whether a *cpxR* or *nlpE* mutation had any effect on virulence gene expression in response to host pathogen interaction. Fusions of the *LEE* encoded genes that are not in operons should also be constructed to determine whether the Cpx response has an effect on their expression as well as to examine their temporal expression patterns. The pJW15 *lux* fusions in EPEC provide a powerful tool to analyze virulence gene expression in the presence of host cells and may allow for the dissection of EPEC virulence regulation.

Finally, as the Cpx response could aid in the folding or degradation of the type III secretory components, it would be extremely useful to determine which components of the TTSS are periplasmic and detail the protein interactions of the apparatus to better understand its construction. Recently, the periplasmic interactions of the type II secretory apparatus of *Erwinia chrysanthemi* have been detailed using a yeast two-hybrid analysis (Douet *et al.*, 2004). A similar approach in EPEC would likely be able to dissect the interactions of the Esc and Sep proteins of EPEC. Periplasmic proteins of the type III secretory apparatus could be identified through creating fusions with alkaline phosphatase, an enzyme only active in the periplasm. As well, introducing epitopes, such as HA tags, without altering reading frame, onto the C-terminus of type III secretory system components could allow for detection of extracellular components of the TTSS through immunofluorescence. The proteins associated with either the periplasm or the periplasmic face of the IM could also be identified through immunofluorescence of a spheroplasted sample of cells, provided the spheroplasting does not disrupt protein interaction. Improved characterization of the EPEC TTSS would lead to further insight about how the Cpx pathway could be involved with its assembly.

In conclusion, this study has been successful in that it has implicated the Cpx system in EPEC pathogenesis, suggesting both transcriptional and post-translational regulatory effects. While the exact nature of the mechanism involved in Cpx-dependent regulation of virulence was not determined, this is the first description of the function of the Cpx two-component system in EPEC. Combined with recent data implicating the Cpx response in the folding of virulence determinants and the regulation of pathogenesis in *Salmonella* and *Shigella*, further study of the Cpx response in EPEC is bound to further elucidate the role stress response systems play in pathogenesis.

6. Appendix

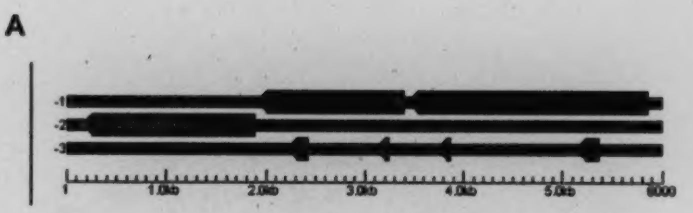
6.1 Attempts to delete a putative EPEC restriction system

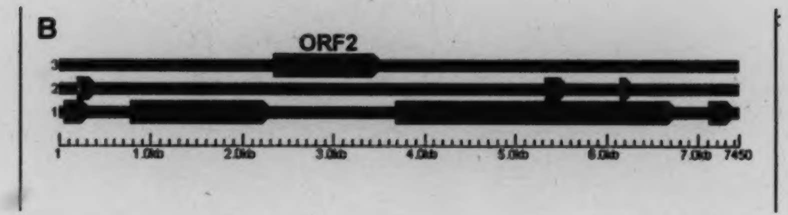
One of the major obstacles encountered during this study was the difficulty in genetically manipulating EPEC. One possible explanation for this could be the presence of a restriction system. Interestingly, fewer problems have been encountered in the manipulation of EHEC despite the degree of similarity between the organisms. This may partially be due to differences in restriction systems (see below).

Several different types of restriction modification systems exist and they are grouped based on enzymatic and target sequence properties, as well as the site of cleavage relative to the target sequence (Murray *et al.*, 2002). The majority of restriction systems in *E. coli* are of the type I group and consist of a hetero-oligomeric complex consisting of a methylase component and a DNA specificity component as well as an independent restriction endonuclease (Barcus *et al.*, 1995, for a review see Murray, 2002). The genes encoding these components are named ***hsdR*** (restriction endonuclease), ***hsdM*** (methylase), and ***hsdS*** (specificity component). ***hsdR*** is generally found upstream of ***hsdMS***, but is transcribed from a separate promoter (Murray *et al.*, 2002). Since the restriction endonuclease is functionally and transcriptionally separate from the methylase complex, an insertional inactivation in the ***hsdR*** gene, encoding the restriction endonuclease, should alleviate restriction while leaving the methylation system intact (Barcus and Murray, 1995).

As the EPEC sequence was not yet published, the first attempt to inactivate the EPEC ***hsdR*** gene was performed by identifying a putative region of the EHEC chromosome that had high homology to the K-12 ***hsdMRS*** region and was near the ***serB*** locus, where the K-12 system is located (Barcus and Murray, 1995). The genes ECs5308, ECs5307, and ECs5306 are homologous to the *hsdR*, *hsdM*, and *hsdS* genes, respectively (Accession AP002569.1) The orientation of the genes is presented in Figure 6.1. Primers were designed to amplify 2kb of the putative EHEC *hsdR* sequence and when *S. flexneri* or EHEC strain O157:H7 chromosomal DNA was used as a template the target was easily amplified. However, no product was ever obtained using EPEC chromosomal DNA as a template. Shortly thereafter the partial EPEC genome sequence was made available and an examination of the sequence searching for DNA homologous to the EHEC *hsdRMS* region failed to yield any strong matches, explaining why the PCR amplification attempts had failed. The sequences of all currently identified *hsdR* genes identified in GenBank were used to search for homology in the partial EPEC genomic sequence. A candidate *hsdR* gene was identified on the Epath140c01.q1cb segment of the sequence ((EPEC genome available for BLAST search at http://www.ncbi.nlm.nih.gov/genomes/framik.cgi?db=genome&gi=5171 but not available for download by Entrez). The putative EPEC restriction genes have a high degree of homology to the EcoR124I restriction system, a type IC system which is usually plasmid borne. Further analysis of this region identified putative ORFs with high homology to the *hsdM* and *hsdS* genes of the EcoR124I system. Restriction systems of this type consist of *hsdM* and *hsdS* under the control of a single promoter and *hsdR* under the control of a separate promoter (Murray *et al.*, 2002). The orientation of the putative EPEC *hsdRMS* genes is given in Figure 6.1 B. Primers designed to amplify a 2kb stretch of the putative EPEC *hsdR* gene successfully produced a product, which was cloned into pUC19 (pJW9), and a *cat* cassette was inserted into the middle of the product (pJW10).

Figure 6.1. Putative EPEC and EHEC restriction-modification loci. BLAST analysis identified a locus in EHEC (A) with homology to type I restriction genes *hsdR* (*ECs5308*), *hsdM (ECs5307)*, and *hsdS (ECs5306)*. Analysis of the partially sequenced EPEC genome identified an ORF with homology to a type IC *hsdR* gene (ORF3) (B). Further analysis identified two upstream ORFs with homology to type IC *hsdM* and *hsdS* genes (ORF2 and ORF1 respectively).


A
-1
-2
-3
1
1.0kb
2.0kb
3.0kb
4.0kb
5.0kb
6000


B
ORF2
3
2
1
1
1.0kb
2.0kb
3.0kb
4.0kb
5.0kb
6.0kb
7.0kb
7450

Primers that amplified approximately 100 or 1000 bases of the putative *hsdR* gene on either side of the *cat* cassette were used to generate PCR products that were electroporated into E2348/69 strains carrying one of the lambda *red* recombination system plasmids pKD46 or pTP223 (Datsenko and Wanner, 2000; Murphy and Campellone, 2003). This approach failed to yield any recombinants. pJW10 was electroporated into E2348/69 in order to achieve methylation by the host modification system, then isolated, digested to isolated the 2.8kb fragment containing the *hsdR:cat* insertion, and gel purified before electroporation into E2348/69 carrying either pKD46 or pTP223. This again failed to yield any recombinants. An unsuccessful attempt was made to move the *hsdR:cat* insertion into pCVD442, a *sacB* containing suicide vector. The *hsdR:cat* insertion was also moved into pHSG415, a temperature-sensitive, partitioning deficient gene replacement vector, but attempts to introduce this construct into E2348/69 proved unsuccessful (White *et al.*, 1999).

Future attempts to generate the *hsdR* mutant might involve using a kanamycin cassette instead of the *cat* cassette in using the lambda *red* system, as inactivation of *cpxR* with a kanamycin cassette has been successful in EPEC using the lambda *red* system, but all attempts using the *cat* cassette have failed (A.Z. Nevesinjac and T.L. Raivio, unpublished). Alternatively, an *hsdR* deletion could be created in pHSG415, then introduced into the EPEC chromosome as this plasmid has been used successfully to create a *tir* deletion in EPEC (DeVinney, 2003). The heat shock step used to introduce the pJW15 based vectors into EPEC should be used to improve transformation frequency and increase the likelihood of obtaining an *hsdR* mutant.

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